ANTI-CANCER ACTIVITY OF SIDDHA DRUG "*ASHTA BAIRAVA CHENDURAM*" AGAINST ORAL SQUAMOUS CELL CARCINOMA [OSCC] THROUGH IN- VITRO MODEL

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ENDORSEMENT BY THE HOD AND PRINCIPAL OF THE INSTITUTION

This is to certify that the dissertation entitled **"Anti-Cancerous Activity of Siddha Drug *Ashta Bairava Chenduram* against Oral Squamous Cell Carcinoma [OSCC] Through In-Vitro Model"** is a bonafide work carried out by **Dr.V.Kaviya** under the guidance of **Prof. Dr.V.Velpandian M.D(S), Ph.D.,** Post Graduate Department of Gunapadam, Govt. Siddha Medical College, Chennai - 106.

Seal and Signature of the HOD **Seal and Signature of the Principal**

Date:

Place: Chennai

ABBREVATION

ABC	*Ashta Bairava Chenduram*
ANOVA	Analysis of Varience
APL	Above PovertyLine
ASTM	American Society for Testing And Materials
ATRA	Arsenic Trioxide and all trans Retiinoic Acid
AYUSH	Ayurveda, Yoga, Unani, Siddha, Homeopathy
BUN	Blood Urea Nitrogen
CCD	Charge Coupled Device
CR	Complete Remission
CT Scan	Computerized Tomography
DDPH	2, 2 –diphenyl -1- picrylhydrazy
DHA	Docosahexaenoic acid
DMBA	7, 12-Dimethyl benzaanthacene
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonuclic acid
DTNB	Dithionitro-bis-benzoic acid
EBV	Epstein – Barr Virus
EDTA	Ethylene Diamine Tetra Acetic acid
FACS	Fluorescence Activated Cell Sortur
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FDA	Fine Needle Aspiration
FTIR	Fourier Transform Infrared Spectroscopy

GPx	Glutothione Peroxidase
GSH	Reduced glutathione
Gy	Gray Unit
HPV	Human Papilloma Virus
IARC	The international agency for research on cancer
IC Dose	Inhibitory of Inhibitor
ICP-OES	Inductively Coupled Plasma Optic Emission Spectrometry
KB	Skin Cancer Cell
LD_{50}	Lethal Dose $_{50}$
LDL	Low Density Lipoprotein
MIR	Medical International Research
MTT	Microculture Tetrasolium Test
NBS	N- Bromosuccinimide
NCCS	National Centre for cell Science
NIHFW	National Institute Of Health and Family Welfare
OECD	Organisation for Economic Co-operation Development
OSCC	Oral Squamous Cell Carcinoma
PBS	Phosphate buffered saline
PCV	Packed Cell Volume
PET	Positron Emission Tomography
RBC	Red Blood Corpuscles
ROS	Review of Symptom / Systems
SAIF	Sophisticated Analytical Instrument Facility
SEM	Scanning Electron Microscope
SGOT	Serum Glutamic Oxaloacetic Transaminase
SRB	Sulphorohodamine
TBA	Thiobarbituric acid

TBARS	Thiobaraituric Acid Reactive Substances
TGL	Triglycerides
TPA	12-O-Tetradicannyl –Phorbol-13-Acetane
VLDL	Very Low Density Lipoprotein
WBC	White Blood Corpuscles
WHO	World Health Organisation
XRD	X-ray power diffraction

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CERTIFICATE BY THE GUIDE

This is to certify that the dissertation entitled **"Anti-Cancerous Activity of Siddha Drug *Ashta Bairava Chenduram* against Oral Squamous Cell Carcinoma [OSCC] Through In-Vitro Model"**is submitted to The Tamilnadu Dr.M.G.R. Medical University in partial fulfillment of the requirements for the award of degree of M.D (Siddha) is the bonafide and genuine research work done by **Dr. V. Kaviya** under my supervision and guidance and the dissertation has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar title.

Date: **Seal and Signature of the Guide,**

Place: Chennai

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled "**Anti-Cancerous Activity of Siddha Drug *Ashta Bairava Chendhuram* against Oral Squamous Cell Carcinoma [OSCC] Through In-Vitro Model"** is a bonafide and genuine research work carried out by me under the guidance of **Prof**. **Dr.V.Velpandian M.D(S), Ph.D(S).,** Post Graduate Department of Gunapadam, Govt. Siddha Medical College, Arumbakkam, Chennai-106 and the dissertation has not formed the basis for the award of any Degree, Diploma, Fellowship or other similar title.

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1. INTRODUCTION

During the last decade, traditional medicinal systems are gaining more importance when compared with conventional medicinal systems of the world. One such is the Siddha system. The Siddha system of medicine was introduced by the ancient scientists "Siddhars'' who were the spiritual adepts, super human beings with high culture and intellectual abilities.

The main goal of the siddha medicine is

- To make one mentally and physically perfect
- To make body imperishable
- To promote longevity

Siddha system is found to be a developed system of medicine yet to be analysed and adopted, under which the science can be understood by the chemistry of exhaustive complicated preparation of medicines which may not be even imagined in ancient days. Heaped up theories with lavish treatment protocols were provided by Siddhars which are yet to be validated safe and effective to satisfy the progressing scientific society.

There are lot of diseases classified under Siddha system in which "*puttru*" is a life-threatening malignant condition. Based on the sites of malignancies, they are named as *puttru*/*kanna puttru* are the malignant conditions of the oral cavity.

Symptoms of *kanna puttru* as explained in Siddha text are as follows;

- Whitish plaques beneath the tongue
- Shooting pain in the ear along with head ache
- Drooling of saliva
- Nausea
- Hyperhidrosis
- Numbness of occipital region [1]

Literary evidences have a classic collection of herbal, mineral, herbo- mineral medicines for Carcinoma, with safety and efficacy yet to be scientifically validated. Several anti-cancerous drugs are now in practice in Siddha medicine such as *Chithiramoola kuligai*, *Saaranaive kudineer*,etc., Apart from the internal medications, Siddhars performed an external procedure called "cauterisation" for treatment of cancerous growth[2].

The above mentioned condition "*puttru*" may be compared to carcinoma of modern age and so *kanna puttru* can be compared with oral carcinoma.

Oral Squamous Cell Carcinoma [OSCC]

Oral Squamous Cell carcinomas (OSCC) are cancers originating from the squamous epithelium in the oral cavity. Locations include the lip, tongue, buccal mucosa, labial mucosa, floor of the mouth, gingiva, hard palate and soft palate[3].

Carcinoma is found to be one of the leading fatal and life threatening conditions worldwide. According to WHO , there were 14 million new cancer cases , 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide.[4] The overall age standardised cancer incidence rate is almost 25% higher in men than in women, with rates of 205 and 165 per 1,00,000 respectively. Expected rise of new cases over the next 2 decades will be about 70% (from 14 million in 2012 to 22 million) [5].

Around one third of cancer deaths are due to 5 leading behavioural and dietary risks:

- High body mass index
- Lack of physical activity
- Tobacco use
- Alcohol use.

Tobacco use is the single most important risk factor for cancer causing about 20% of global cancer deaths and around 70% of global lung cancer deaths [6].Another major risk category is oral cancer. Oral cancers are a group of cancers commonly referred as head and neck cancers.

According to American cancer society, men face twice the risk of developing oral cancer as women and men who are over age 50 face the greater risk.It is estimated that over 40,000 people in the U.S received a diagnosis of oral cancer in 2014.

An epidemiologic and clinical review published in Journal of community health, reveal that there is a high prevalence of oral cancer in Indian community. Statistical analysis shows the prevalence is about 1.12%[7].

Another study was done by the Department of Public health and Primary care, University of Cambridge in India about the challenges of oral cancer burden in India. This study says Oral cancer ranks in the top three of all cancers in India, which accounts for over 30% of all cancers reported in the country. In India age-adjusted rates of oral cancer is high that is 20 per, 1,00,000 population & accounts for over 30% of all cancers in the country[8].

Experts of National Institute of Health & Family welfare (NIHFW) prepared reports recently which mainly focus on the harmful effects of gutka, which clearly specifies that India alone accounted for 86% of total oral cancer across the world. Chewing tobacco and gutka contribute to 90% of oral cancer cases in the country [9].

The most common risk factors for oropharyngeal cancer include the following:

- Being infected with human papillomavirus (HPV). The number of oropharyngeal cancers linked to HPV infection is increasing.
- A history of smoking for more than 10 pack years.
- Heavy alcohol use [10].

Treatment for Oral carcinomas majorly includes chemotherapy and radiation therapy. Chemotherapy is the use of anti-cancer (cytotoxic) drugs to destroy cancer cells. Chemotherapy drugs work by disrupting the growth of cancer cells. The most commonly used Chemotherapy drugs for head and neck cancer is Cisplatin.

Other chemotherapy drugs that may be used
include Carboplatin, Docetaxel (Taxotere),Gemcitabine and Fluorouracil (5FU)[11].

Adverse effects of chemotherapy

Myelosuppression, nausea, vomiting, hyperuricaemia, alopecia etc

Present studies stress on the fact of increasing toxicity due to Chemotherapy, specifically in head and neck cancer such as Mucositis (painful inflammation and ulcerations of the mucous membranes) and Xerostomia (dryness of mouth) as a major manifestation of chemotherapy and radiation therapy respectively.There are additional studies under way to address the long-term side effects as well as the acute toxic effects of combination therapy to the head and neck region[12].

The adverse side effects of chemotherapy is a rising burden for the Cancer affected community which affects their routine day to day activities to a great extent both mentally

and physically, which naturally leads to a urge of searching safe treatments with less side effects.[13] And thus, there is a need of searching traditional healing in a smoother way. In addition to herbal treatments, a huge number of Herbo-mineral and metallic preparations are found in Siddha literatures, one among those is "*ASHTA BAIRAVA CHENDURAM*" ,which is a red oxide product with the ingredients Red Orpiment, Yellow Arsenic Trisulphide, Magnetic Oxide of Iron, Hydragyrum Subchloride, Red Sulphide of Mercury, Hydragyrum, Sulphur, White Arsenic and fresh juices of*Acalypha indica, Piper betle, Gossypium herbaceum, Enicostema axillare, Ocimum sanctum.*

Researches bring into limelight that the above compounds of Arsenic and Sulphur are found to have Anti-oxidant activity and Anti-tumour activity to an extent that they could be used in conditions where the affected are intolerable to chemotherapy[14].

- Anti-tumour activity of Arsenic compounds
- Anti-oxidant activity of Arsenic trioxide
- Synthesis and Anti-tumour Activity of sulphur-containing 9-Anilinocridines[15]

Piper betle has cytotoxic activity as well as microbial activity, *Acalypha indica* possess Anti- oxidant and Anti- cancer activities, *Acalypha indica* possess potent Anti-cancerous property, *Ocimum sanctum* possess antioxidant, anticancer, chemopreventive, radio protective, immunomodulatory activities, *Enicostemma axillare* shows greater activity on HeLa cell line and that mean *Enicostemma axillare* can be used in anticancer activity [15]. Another study reveals that *Enicostemma axillare* is also having anti-oxidant activity[16]. *Gossypium herbaceum*is found to have Anti-viral activity and Anti –Bacterial Activity which is highly essential in treating HPV induced Oral Carcinoma[16][17][18][19][20][21][22].

Thus, the drug *Ashta Bairava chenduram* is taken for analysis of Anti-cancer, Anti-tumour, Anti-oxidant activities that it can be evolved into a better treatment for Cancer compared to chemotherapy.

2. AIM AND OBJECTIVE

AIM:

To justify the ancient Siddha drug for management of oral cancer with its ultimate formulation and give good progress to the people affected by oral cancer. The purpose of the present study was aimed to evaluate the activity of *Ashta Bairava Chenduram* for its efficacy in managing Oral Cancer through pre-clinical aspects. In the present medical world, there is a need for proper treatment for Oral Cancer. The aim of this study is evaluation of a new drug for the management of oral cancer.

OBJECTIVES:

The key objectives of the study are:

- Having a collective review of the literature.
- Preparing the drug according to Siddha classical text.
- Subjecting the drug to physico-chemical standardization.
- Analyzing the drug chemically for detection of acid and basic radicals.
- Focusing the drug for analytical assessment.
- Studying the toxicity profile of *Ashta Bairava Chenduram* according to OECD guidelines.
- Determining the pharmacological activity (*In Vitro* Anti-cancer activity) of *Ashta Bairava Chenduram* .
- Analyzing all the above study results to evaluate the benefits of *Ashta Bairava Chenduram* .

3. REVIEW OF LITERATURE

3.1 SIDDHA REVIEW

SIDDHA ASPECT OF *MANOSILAI* (REALGAR):

Synonyms:

Realgar

Chemical names:

Arsenic Disulphidum, Bisulphuret of Arsenic

Types:

Manosilai (Bisulphuret of Arsenic) is of two types

1. *Piravi sarakku* – It is naturally available.

2. *Vaippu sarakku* – It is obtained by adding 5 parts of Arsenic trioxide and 3 parts of Sulphur.

General properties:

“..... கொடிய குஷ்டம்

காய்ச்சல்நடுக்கலஜ கல்லியிரைப் புச்சிலந்திப்

பேசறும னோசிலைக்குப் பேசு”.

-பதார்த்த குண சிந்தாமணி.

It has got body strengthening and rejuvenating properties. Its potency is good. This is effective in the treatment of leprosy, fever with chills, asthma, eye diseases, urinary tract infections, *kapha* diseases and cervical adenitis.

Method of purification:

Manosilai is triturated with any one of the following for 3 hours, Ginger juice, lemon juice or butter milk, it is then dried to get purified form.

Medicinal uses:

It is mostly not used alone but in combination with other drugs, pills and oil. The oil is effective in the treatment of fistula. The pill is effective in the treatment of fever with chills.

Other medicines:

Kasthuri karuppu, Vishnu Sakkara maththirai, Gandhaga urundai, Paashana maththiarai, Bramananda bairavam, Sivanar amirtham, Santhirothaya maththirai[23A].

3.2 MODERN ASPECT OFREALGAR:

Realger, α-As_4S_4 is an Arsenic Sulphide mineral, also known as “Ruby Sulphur”. It is orange-red in colour, melt at 320^0C,and burns with the bluish flame releasing fumes of Arsenic and Sulphur. It is a photosensitive mineral and will alter to Para Realger upon prolonged exposure to light. It has an Arabic name *Rahj al ghar* which means "powder of the mine."

Other names: Ruby Sulphur, Ruby of Arsenic.

Physical Properties:

Formula	-	As_4S_4 or AsS
Colour	-	Red to yellow-orange
Density	-	3.56
Diaphaneity	-	Transparent
Specific gravity	-	3.56
Melting point	-	320 °C
Molecular Weight	-	106.99 gm
Refractive index	-	2.538
Luminescence	-	Non-fluorescent
Lustre	-	Sub Metallic
Streak	-	Orange

Electrical Properties:

Electron Density - 3.30 gm / cc

Fermion Index - 0.0022478773

Boson Index - 0.9977521227

Radioactivity - 0 GRapi (Gamma Ray American Petroleum Institute Units) [24]

Uses:

The Chinese name for Realgar is *Xionghuang*, literally 'masculine yellow'. It was used to repel snakes, rats, weeds and insects, as well as being used in Chinese medication. The ancient Greeks called it as "*Sandaracha*". It is used in combination with Potassium Chlorate to make a contact explosive known as "red explosive" for some types of torpedoes[25].

SIDDHA ASPECTOF *THAALAGAM* (ORPIMENT):

Synonyms:

Orpiment

Chemical names:

Yellow Arsenic Trisulphide, Trisulphuret of Arsenic

Other names:

Peethagi, Aalmbi, Paluppu, Kothantham, Maalam, Arithaaram, Kaalpuththi, Ponvarni, Manjal varni, Maaldevi and Arithalam

Types:

Depending upon the colour, appearance and properties, *Thaalagam* has beenclassified into four types.

1. *Sivappu Aridharam* (Red Orpiment)
2. *Madal Aridharam*
3. *Pon Aridharam* (Gold Orpiment)
4. *Karattu Thalagam*

General properties:

"தாளகத்தின் பேருரைக்கதத் தாலுகவுள் நோய்குஷ்டம்

நீளக் குளிர்காய்ச்சல் நீடுகபம் - நாளாகங்கொள்

துஷ்டப் பறங்கிப்புண் சூழழுகண் மண்டைநோய்

கிட்டப் படுபமா கிளத்து"

-பதார்த்த குண சிந்தாமணி.

It is effective in the treatment of skin diseases, diseases of head and tongue, fever with chills, *kapha* diseases, urinary tract diseases, and venereal focus ulcer in the urethra.

Actions:

Expectorant, antipyretic, convalescent, tonic, emetic

Other medicines:

Thalaga ennai (Virana sanjeevi thailam) - Heals chronic ulcers

Signs and symptoms of yellow Arsenic poisoning:

Yellow Arsenic if not prepared properly, the preparation will be toxic. The following symptoms are seen, burning pain of the stomach, gastritis, hoarseness of voice, nasal bleeding, bleeding from the nail buds, itching over the head and redness in the tip of the hairs, mental disorders, lower abdominal swelling and throbbing pain in the lumbar region, bronchitis and sciatica.

Antidote:

Root Bark of Ceylon lead wort (*Plumbago zeylanica*)	_	8.75gm
Pepper (*Piper nigrum*)	–	8.75gm

These are added together and made decoction. Culinary salt (4.37gm) is then added and the mixture is taken twice daily for 21 to 42 days[23B].

MODERN ASPECT OF ORPIMENT:

Orpiment is a deep orange-yellow colored Arsenic Sulfide mineral. Its formula is As_2S_3. It is formed by sublimation of Arsenic (60.90%) and Sulphur (39.10%). It takes its name from the

Latin Auripigmentum (Aurum − Gold + pigmentum − pigment) because of its deep-yellow color.

Synonyms:

Arrhenicum Operment, Oropiment, Yellow Arsenic, Yellow Ratsbane, Auripigment, Arsenicum flavum

Physical Properties:

Colour	-	Lemon yellow, Orange yellow
Density	-	3.49 - 3.56, Average = 3.52
Molecular Weight	-	246.04 gm
Melting point	-	300 °C to 325 °C
Diaphaneity	-	Transparent to translucent
Fracture	-	Sectile
Crystal Habit	-	Foliated
Hardness	-	1.5-2 - Talc-Gypsum
Luminescence	-	Non-fluorescent
Luster	-	Pearly
Streak	-	Pale yellow
Electron Density	-	3.23 gm / cc
Specific Gravity	-	3.49 gm / cc
Fermion Index	-	0.0029323747
Boson Index	-	0.9970676253
Photoelectric	-	44.66 barns / electron
Radioactivity	-	0 GRapi (Gamma Ray American Petroleum Institute Units)

Chemical Properties:

Formula - As_2S_3

Elements - As, S

Common Impurities - Hg, Ge, Sb [26]

Uses:

Orpiment was traded in the Roman Empire and was used as a medicine in China. It has been used as a fly poison and to tip arrows with poison. Because of its Golden color, it was used by alchemists, both in China and the West, searching for a way to make Gold[27].

SIDDHA ASPECT OF *KAANTHAM* (MAGNETIC OXIDE OF IRON):

Chemical name – Magnetic oxide of Iron

Other names:

Sivaloga Sevagan, Tharanikku Naatham, Sootha Angusam, Navaloga Thurati, Kaayachithikku Paathiravan, Murugan Puranam.

Types:

There are five varieties of *Kaantham*

1. *Kal kaantham (Piramugam)*
2. *Oosi kaantham (Kambagam)*
3. *Pachai kaantham (Karshagam)*
4. *Arakku kaantham (Dhiravagam)*
5. *Mayir kaantham (Romagam)*

General properties:

“காந்தத்தாற் சோபைகுன்மங் காமிலமே கம்பாண்டு

சேர்ந்ததிரி தோடவெட்டை சீதங்கால் - ஓய்ந்தபசி

பேருதரங் கண்ணோய் பிரமியநீ ராமையும்போம்

ஓரினிறை யாயூளுறும் உன்”.

-பதார்த்த குண சிந்தாமணி.

In general, the *Kaantham* has got the similar properties as Iron. However, it is considered that *Kaantham* issuperior to Iron in many aspects. This is very effective in the treatment of swelling, ulcer, jaundice, venereal diseases, *kapha vatha* diseases, leucorrhoea, dyspepsia, gonorrhea, anasarca, eye diseases and splenomegaly. It also increases one's life span.

Method of purification:

Magnetic oxide if Iron - 35 gm

Root bark juice of *Ponnavarai* (*Cassia auriculata)* - 210 gm

Magnetic oxide of Iron is soaked in root bark juice of *Ponnavarai* and isolated from morning to evening for ten days. Then it is dried for two days without adding the juice. This process is repeated twice and washed to obtain purified Magnetic oxide of Iron [23C].

MODERN ASPECT OF MAGNETIC OXIDE OF IRON:

Other names:

Iron (II, III) oxide, Iron (II) Iron (III) oxide

Magnetite is an important ore of Iron. It is a natural Iron oxide Magnet, hence the name, giving it a very nice distinguishing characteristic. It is the most magnetic of all the minerals on Earth. In Magnetite, Fe3O4, the A metal is Fe +2 and the B metal is Fe +3; two different metal ions in two specific sites. This arrangement causes a transfer of electrons between the different ions in a structured path or vector.

Distribution:

Magnetite is sometimes found in large quantities in beach sand. Such mineral sands / black sands are found in various places such as California and coastal area of New Zealand.

Properties:

Colour - Black to grayish

Molecular formula - Fe_3O_4, $FeO.Fe_2O_3$

Molar mass	-	231.533 g/mol
Appearance	-	solid black powder
Density	-	5 g/cm3
Melting point	-	1538 °C
Refractive index (nD)	-	2.42 [1]
Luster	-	Metallic
Tenacity	-	Brittle
ID Mark	-	Ferromagnetic
Solid Density	-	5.1(gm/cm3)
pH	-	7
Transparency	-	Opaque
Hardness	-	5.5 to 6.5
Fracture	-	Conchoidal
Specific Gravity	-	5.17 to 5.18
Crystal System	-	Isometric

Medicinal Uses:

Nanoparticles of Fe_3O_4 are used as contrast agents in MRI scanning. Ferumoxytol is an intravenous Fe_3O_4 injection for treatment of anemia resulting from chronic kidney failure.

Other Uses:

Magnetite is used as a black pigment and is known as C.I pigment black 11. It is used as a catalyst in the Haber process and in the water gas shift reaction. It is used as a pigment for Magnetic applications, polishing compounds and cosmetics. [28]

SIDDHA ASPECT OF *POORAM* (CALOMEL):

Synonyms:

Calomel

Chemical names:

Hydragyrum Subchloride

All though the calomel does not find a place in the list of 64 *P*aadanaas, it is considered as one among them by the medical practitioners. It is a combination of *Rasam* (Mercury) and salt.

Method of Preparation of *Pooram:*

Sulphur 67.2 gm is melted in a mud pot and Mercury 33.6 gm is added to it and kindled well to get a black coloured powder. Brick powder is placed up to the half of the level of a pot. Culinary salt (Nacl) – 650 gm is placed over it. Mercury and Sulphur mixture is placed over the salt and sealed with mud pasted cloth. It is burnt for 12 hours with *Kaadakkini*. After it is cooled the Mercurous Chloride is found deposited on the upper pot and the same are collected.

General properties:

"இடைவாத சூலை எரிசூலை குன்மந்

தொடைவாழை வாதமாஞ் சோணி - யிடையாதோ

வொக்குரச கற்பூர மொன்றே யாளவொடுநல்

இக்கு வெல்லத்தேழு நாளீ".

-பதார்த்த குண சிந்தாமணி.

It cures throbbing pain, pain in the lumbar region, burning sensation, ulcer due to disorder of *vatha* humour, hepatomegaly, pyrexia, jaundice, bacillary dysentery, dropsy, chronic ulcers, venereal diseases, vomiting, diarrhea, worm infestation, rheumatism, itching, constipation, scabies and headache.

Taste – salty

Potency – hot

Action – Laxative, tonic, antiseptic and diuretic

Purification method of Calomel:

The poultice made of Betel leaf (*Piper betle*) and Peper (*Piper nigrum*) each 8.75 gm are taken and dissolved in 1.3litre of water. Calomel (50 gm) is tied with the cloth and immersed in the liquid form the cross bar and heated. After the water is reduced to ¾ of its volume. The Calomel is taken out, washed with water and dried to get purified Calomel.

Toxic symptoms of Calomel:

Multiple red boils appear on the face, acne formation, ulcers in the chest, mouth and tongue, diarrhea, dysentery, scrotal swelling, and ulcer in the uvula.

Antidote:

Black musali tubers (*Curculigo orchiodes)* - 8.75 gm

Indian penny wort root (*Centella asiatica)* - 8.75 gm

Root of sessile plant (*Alternanthera sessilis)* - 8.75 gm

Beetle killer (*Cleodendrum serratum)* - 8.75 gm

All these ingredients are mixed together and boiled to make a decoction. It is used twice a day for two or three weeks with suitable diet restrictions.

Other preparations:

Rasa Karpoora Kuligai - Vulval Cancer, penile Cancer, chronic ulcer, deep ulcers and syphilis

Poora Kalimbu - Syphilitic ulcer

Poora Ennai - Venereal diseases, ulcers

Poora Podi - Venereal ulcer occurring on penis [23D]

MODERN ASPECT OF CALOMEL:

Mercury (I) chloride is odourless solid and dense white or yellowish-white in colour. It is the principal example of a Mercury (I) compound. It is composed of Mercury and

chlorine (Mercury 84.98 % Chlorine 15.02 %). It is also referred to as the mineral horn quickSilver or horn Mercury.

Properties:

Molecular formula - Hg_2Cl_2

Molar mass - 472.09 g/mol

Molecular Weight - 472.09 gm

Appearance - White solid

Specific - 7.27 gm / cc

Density - 7.150 g/cm3

Melting point - 525 °C (triple point)

Boiling point - 383 °C (sublimes)

Solubility in water - 0.2 mg/100 ml

Hardness - 1.5-2 - Talc-Gypsum

Refractive index - 1.973

Fermion Index - 0.25

Boson Index - 0.75

Radioactivity - 0 GRapi i.e not radioactive (Gamma Ray American Petroleum Institute Units)

Other anions - Mercury (I) fluoride, Mercury (I) bromide, Mercury (I) iodide

Other cat ions - Mercury (II) Chloride [29]

Medicinal properties:

Calomel was used internally to treat yellow fever during its outbreak in Philadelphia in 1793 and also used in the treatment of syphilis, until early 20th century. It used as a laxative and disinfectant. During 18th century American Doctors used Calomel to make

patients regurgitate and release their body from "impurities". It was a common ingredient in teething powders, soaps and skin lightening creams in Britain up until 1954[30].

SIDDHA ASPECT OF *LINGAM* (CINNABAR):

Synonyms: Natural Cinnabar, Vermilion.

Chemical name: Red Sulphide of Mercury.

Other names:

Inkuligam, Raasam, Kadai vanni, Karpam, Kalikkam, Kaanjanam, Kaaranam, Sandagam, Samarasam, Saaniyam, Chendooram, Maniragam, Milechem, Vani andVanni.

Nowadays, The *Lingam* used by us is called as *Jaathi linga paadanam,* grouped under *Vaippu paadanam.*

Preparation of *Vaippu paadanam:*

Rasam (Mercury) – 280 gm

Gandhagam (Sulphur) – 70 gm

Vediuppu (Pottassium nitrate) – 70 gm

Procedure:

Mercury is thoroughly mixed and triturated with Sulphur. Potassium nitrate is then added, placed in a conical flask and burnt for 18 hours, after cooling the red Sulphide of Mercury is collected out.

***Gunam* (Properties):**

It is hard, when it is put into fire it developes smoke; not soluble in water, has no smell and taste and has hot potency.

General properties:

“பேதிசுரஞ்சந்நி பெருவிரண நீரோடுத

காதகடி காசங் கரப்பான்புண் - ணோத

வுருவிலிங்க சங்கதமா யூறுகட்டி யும்போங்

குருவிலிங்க சங்கமத்தைக் கொள்."

-பதார்த்த குண சிந்தாமணி.

It is effective in the treatment of diarrhea, pyrexia, delirium, urticaria, diuresis, tuberculosis, scabies, unknown insect bites, syphilis, leprosy, eczema, skin diseases, throbbing pain and *vatha* diseases.

Method of purification:

Lime juice, cow's milk and the Indian Acalypha juice are mixed together in equal proportion and allowed to fuse Cinnabar so as to get it in a purified potent form.

Other preparation:

Sanda Rasa Parpam - syphilis, arthritis, tremor, delirium and venereal diseases

Padigalinga Chendooram - dysentery, diarrhea, menorrhagia and fever

Saathi Sambera Kuzhambu - diarrhea, nausea, vomiting, syncope, fever and thirst

Sign and symptoms of Cinnabar toxicity:

Dyspepsia, loss of taste, ulcers in the buccal floor, uvula, inner portion of the tongue, larynx and large intestine, foul odour from mouth, discharge of viscous, whitish saliva burning sensation are the toxic symptoms of red Cinnabar.

Antidote:

Nutmeg (*Myristica fragrans)* - 4.2 gm

Cubeb pepper (*Piper cubeba)* - 4.2 gm

Root bark of red cotton tree *(Gossypium arboretum)* - 4.2 gm

Sugar candy - 4.2 gm,

These are mixed together and made into decoction and administered twice daily for 48 days. [23E]

MODERN ASPECT OF CINNABAR:

Cinnabar (red Mercury (II) Sulfide (HgS), vermilion) is the ordinary ore of Hg. It is normally found in a substantial, granular form and is bright scarlet to brick-red in color. It is a chemical compound composed of the chemical elements Mercury and sulphur (Mercury 86.22 % Sulphur 13.78 %).

Formula - Mercury (II) sulfide

Symbol - HgS

Properties:

Molecular formula - HgS

Number - 32

Colour - brownish red and lead-gray

Specific gravity - 8.176

Solubility - Soluble in water,

Molecular Weight - 232.66 gm

Melting point - 580 °C decomp.

Other anions - Mercuryoxide, Mercury selenide, Mercury telluride

Other cations - Zinc sulphide, Cadmium sulphide

Fermion Index - 0.26

Boson Index - 0.74

Radioactivity - 0GRapi i.e not radioactive (Gamma Ray American Petroleum Institute Units) [31]

Toxicity:

It caused shaking, loss of sense, and death. Over exposure to Mercury is called "Mercurialism". This was seen as an occupational disease to the ancient Romans [32].

SIDDHA ASPECT OF *RASAM*:

Synonyms: Mercury orQuickSilver

Chemical name: Hydragyrum

Mercury is comes under the classification of '*Pancha soothaam'*. It has many connotations such has *Sootham, Punniyam, Bharatham, Inimai, Sivasathi, Kesari* etc, according to *Dasangu nigandu.*

Mercury is obtained from its ores in countries like Spain, California, Russia, China and Japan. It is separated from its ore Cinnabar.

Types of Mercury:

Mercury was classified into five types.

1. *Rasam*
2. *Rasendhiran*
3. *Sootham*
4. *Misaragam*
5. *Baaratham*

Properties:

1. Vitalizer
2. Tonic
3. Laxative
4. Diuretic
5. Neutralising *pitham*
6. Silagogue
7. Anti-inflammatory
8. Medicine for venereal disease (*mega nasini*)

Taste: Six tastes dominated by sweet

Potency: Hot and cool (both -speciality)

Special properties of Mercury:

Unlike other drugs Mercury is useful in the treatment of diseases caused by both heat and cold.

***Dhosam* (Impurities) of Mercury:**

It is considered that there are two types of *Dhosam* of Mercury. They are

1. Dhosam

2. Sattai (Kavasam)

In *Dhosam* there are 8 types of impurities in Mercury producing various diseases as shown below

Impurities	**Disease caused by them**
1. Undheenam	*Soolai* (Throbbing pain)
2. Kowdilayam	*Kapala noi* (Diseases of the head)

3. *AnavarthamBiramai* (Manic illness)

4. *SangaramThathu nattum* (Spermatorrhoea)

5. *Sandathvam Sattium* (distress)

6. *Panguthvam Kuttam* (Leprosy)

7. *SamalathvamMoorchai* (Syncope)

8. *SavisthavamSareeraElaippu* (Loss of weight)

Sattai is an another type of classification, there are 7 types of impurities in Mercury which produces various diseases as shown below

Impurities	**Diseases caused by them**

1. *NaagamMoolam* (Haemorrhoids)

2. *VangamTholnoikal* (Skin disease)

3. *MalamArivinmai* (Idiocy)

4. *VidamMaranam* (Death)

5. *AkkiniThaha moham (*Polydypsia)

6. *GiriSattium* (Distress)

7. *SabalamThathunattam* (Spermatorrhoea)

General properties of Mercury:

விழிநோய்கிரந்திகுன்மம்மெய்சூலைபுண்குட்

டழிகாலில்விந்துவினால்அத்தை- வழியாய்

புரியுவிதியாதுபுரியினோயெல்லாம்

இரியுவிதியாதுமில்லை.

Proper use of Mercury as a medicine can able to cures the following diseases they are disease in eyes, syphilis, eight types of ulcers (*gunmam*), throbbing pain (*soolai*), chronic ulcers (*perumpun*), leprosy and Hansen's disease.

Purification and detoxification of Mercury:

- Mercury -35gram
- Brick powder-required quantity
- Turmeric powder- required quantity
- *Acalyphaindica* juice-1.3 lit

Mercury was triturated with finely powdered brick and then turmeric powder for one hour respectively and washed with water. Then Mercury is boiled with the juice of Acalypha indica till the juice completely evaporates. We get purified Mercury.

Preparations of Mercury:

- *Sootha karuppu*

- *Rasa mezhugu*
- *Rasa thailam*
- *Megavirana kalimbu*
- *Rasa kuligai*[23F]

MODERN ASPECT OF MERCURY:

Mercury:

Mercury should not have less than 99.5 percent of Hg. It occurs naturally as a sulphide ore called Cinnabar HgS. It also occurs in small globules disseminated through rocks and as amalgam of Silver and Gold.

Preparation:

It's obtained by roasting Cinnabar in a current of air $HgS+O_2 \rightarrow Hg+SO_2$

The free Mercury gets liberated it may be either purified by volatilization or chemically by dropping Mercury into a column of dilute Nitric acid for removing basic impurities.

Properties:

It has shinning silvery white in nature. Heavy liquid easily divisible into globules and extremely mobile it easy volatilizes on heating. It boils at 359.58°C.

Almost insoluble in water, alcohol and HCl. it dissolves in cold and dilute Nitric acid, giving mercurial nitrate and Nitric oxide.

$$6Hg+8HNO_3 \rightarrow 3Hg_2 (NO_3)2+2NO+4H_2O$$

Density:

13.581ml at 25°C

Mercurial preparations:

- Mercury with Chalk (Gray powder)
- Yellow Mercuric Oxide (HgO)

- Mercuric Oxide
- Oleated Mercury
- Mercurous Chloride (HgCl-Calomel)

Tests for Purity:

It has been tested for weight per ml (at 25°C is about 13.5g).Non-volatile matter residue at 300°C (not more than 0.02%w/w).

Assay:

An accurately weighed quantity (0.49g) is dissolved in equal parts (20ml) of water and Nitric acid. It is heated gently until the solution become colourless. The solution is then diluted with water (150ml) and sufficiency quantity of Potassium permanganate is added till a permanent pink colour is produced. A trace of Ferrous sulphate to discharge pink colour is added. Then the solution is titrated with standard 0.1N Ammonium thiocyanate (1ml of 0.1N Ammonium thiocyanate =0.01003g), using Ferric Ammonium sulphate as indicator. The temperature during the titration should not exceed above 20°C.

$3Hg+8HNO_3 \rightarrow 3Hg(NO_8)2+4H_20+2NO\uparrow$

$Hg(NO_3)2+2NH_4SCN \rightarrow 2NH_4NO_3+Hg(SCN)2$

Uses:

It is a pharmaceutical aid and for preparing Mercury with chalk .Formerly metallic Mercury found use as such therapeutically as a cathartic and parasticide. But it is more used as such, as it has been extremely poisonous and prolonged inhalation of even very minimal amounts of Mercury prove fatal. Almost all the salts of Mercury with the exception of the Sulphide, has been poisonous.

1. Mercury with chalk (Grew powder)

- It is having 31 -35% w/w of Mercury and 62-70% w/w of $CaCO_3$
- It is used as a purgative (Dose 60-300mg)

2. Yellow mercuric Oxide (HgO)

- ❖ It is having not less than 99.5% HgO. It is used as a mild anti-septic, as anti-infective and anti- bacterial agents.

3. Mercuric Oxide:

- ❖ It contains not less than 95% but not more than105% w/w of the stated amount of yellow Mercuric oxide
- ❖ It is used in ophthalmology, 1% ointment to treat mild inflammatory conditions for the treatment of blepharitis and conjunctivitis.

4. Oleated Mercury:

- ❖ It has the equivalent of 20% of yellow Mercuric oxide
- ❖ It is used as an anti-infective.

5. Mercuric chloride (HgCl) (Calomel):

- ❖ It is being not less than 99.6% of HgCl
- ❖ It has been used for centuries as a cathartic but recently it is replaced by other drugs. [33]

Calomel has been insoluble in gastric juice and has been not absorbed from the stomach. It gets absorbed in the intestine by the alkaline pancreatic juice where it slowly gets dissociated into Mercury and irritant Mercuric compounds which have been exerting a cathartic action [34].

***SIDDHA ASPECT OF GANDHAGAM* (SULPHUR)**

Chemical name – Sulphur

Other names:

Kaarizhain naatham, Parai veeriam, Atheetha prakaasam, Peejam, Sakthi, Sakthi peesam, Selvi vindhu, Naatham, Naatram, Deviuram

General properties:

Gandhagam is bitter and astringent in taste. Its actions are laxative, tonic and antiseptic. It increases the various secretions of the body including skin. When used in high doses, it causes diarrhea.

Types:

Gandhagam is divided into four types depends upon their colour, appearance and properties.

1. White colored Sulphur
2. Red colored Sulphur
3. Golden yellow colored Sulphur
4. Black colored Sulphur.

In addition, gooseberry Sulphur and stick Sulphur (*Vaana gandhagam)* have been mentioned in most of the text books of ancient Siddha medicines. Gooseberry Sulphur is one which is often used in medicinal preparations.

Gooseberry Sulphur (*Nellikai gandhagam):*

"நெல்லிக்காய்க் கந்திக்கு நீள்பதினெண் குட்டமந்தம்

வல்லை கவிசைகுன்ம வாயுகண்ணோய் - பொல்லா

விடக்கடிவன் மேகநோய் வீறுசுரம் பேதி

திடக்கிரக ணீகபம்போந் தேர்".

-பதார்த்த குண சிந்தாமணி.

It is used in the treatment of 18 types of skin diseases, liver enlargement, abdominal distension, eye diseases, chronic venereal diseases, chronic diarrhea, gastric ulcer, poisonous bites, fever, and chronic dysentery.

Method of purification:

Sulphur is placed in an Iron spoon. A small quantity of cow's butter is added and the spoon is heated till the butter melts, this mixture is immersed in inclined position in cow's milk. This procedure is repeated for 30 times to get purified Sulphur. Each time fresh milk is to be used. [23G]

MODERN ASPECT OF SULPHUR:

Sulphur or Sulphur is a Greek word which means “to burn”. Sulphur is a chemical element with the symbol S. It is a plentiful, multivalent non-metal. It occurs in nature as the pure element and as Sulfide and Sulfate minerals. Sulphur is referred to in the Bible as brimstone (burn stone) in English.

History:

It is discovered by Chinese Before 2000BC and is recognized as an element by Antoine Lavoisier in 1777.

General properties:

Symbol - S

Number - 16

Element category - polyatomic nonmetal

Physical properties:

Phase - solid

Density - 1.96 g·cm−3

Liquid density at M.P - 1.819 g·cm−3

Heat of fusion - 1.727 kJ·mol−1

Heat of vaporization - 45 kJ·mol−1

Molar heat capacity - 22.75 J·mol−1·K−1

Electronegativity - 2.58 (Pauling scale)

Chemical properties:

Solubility - insoluble in water

Vanderwaals radius - 0.127 nm

Ionic radius - 0.184 (-2) nm; 0.029 (+6)

Isotopes - 5

Electronic shell - [Ne] 3s23p4

Standard potential - 0.51 V

Biological role:

Sulphur is a vital component of all alive cells, it is the seventh most plentiful element in the human body by weight, and is used in biochemical processes. The average person takes in around 900 mg of Sulphur per day, mainly in the form of protein. In metabolic reactions, Sulphur compounds serve as both fuels and respiratory materials. Its organic form is present in the vitamins biotin and thiamine. It is an essential part of many enzymes and in antioxidant molecules. [35]

Toxic effects of Sulphur:

Elemental Sulphur is nontoxic, but many simple Sulphur derivatives are, such as Sulphur dioxide (SO_2) and Hydrogen Sulfide are toxic which include neurological effects, disturbance of blood circulation, heart damage, reproductive failure, stomach and gastrointestinal disorder, dermatological effects. [36]

SIDDHA ASPECT OF *VELLAI PAASHAANAM* (WHITE ARSENIC):

Synonyms:

White Arsenic

Chemical names:

Arsenious acid

Vellai Paashaanam is obtained from nature in the land. It is mostly available in combination with other metals such as Sulphur, Iron, and Copper etc.

General properties:

When used in small doses, it stimulates the appetite, improves body strength, removes neurasthenia, reduces fever. It also stimulates the heart, lung, bowels and reproductive organs; improves the resistance to infections. It is generally used in *Unani* medicine for its reproductive function.

Actions:

Appetizer, Tonic, Nervine Tonic, Antipyretic

General properties:

"வெள்ளைப்பா டாணம் விடங்கடிதீ ரும்பூசக்

கொள்ளைச் சுரதோஷங் கோரசந்நி - தொள்ளையுறு

நாசிப்புண் வாய்ப்புண் நனைகிரந்தி போமுண்ண

ஆசிக்கு கும்பமுலை ஆய்."

-பதார்த்த குண சிந்தாமணி.

It has been found to be effective in the treatment of epidemic fever, poison bite, delirium, infections and ulcer of the nose and mouth venereal ulcers, filarial fever, skin diseases and asthma. Its dosage is 4-8 mg.

Method of purification:

Arsenic trioxide 35 gm is powdered and triturated with lemon juice. It is made in to small cakes and dried. This process is repeated for 7 times.

Toxic symptoms of Arsenic trioxide:

Acute poisoning:

Blister, ulcer, pain in the hands and legs finger, swelling of the face, ulcer of the upper lip, vomiting and bad odor, tastelessness, sore throat burning sensation in the stomach, bleeding diarrhea and vomiting, hemetemesis, excessive sweating, thirst, strangury, syncope, convulsion, loss of memory and anasarca.

Chronic poisoning:

Pruritus, eczema, chronic hepatitis, indigestion, swelling of the face, pain in the throat, gastritis, giddiness and diarrhea.

Antidote:

- Cardamom and root of *Bryonia Scabrella* (4.2 gm each) are taken and decoction is made with sugar and Alum. It is taken twice a day for 40 days.

- If *Pitha* exceeds with vomiting, give decoction.
- Give pepper paste for curing the Arsenic trioxide poisoning.
- The *Kalkam* of the Indigo plant root can also be used in Arsenic poisoning, as twice a day [23H].

MODERN ASPECT OF WHITE ARSENIC:

Arsenic trioxide - As_3+ O_2-

Systematic name - Diarsenic trioxide

Other names - Arsenic (III) oxide, Arsenic sesquioxide

Properties:

Appearance - White solid

Molecular formula - As

Molar mass - 197.841 g/mol

Density - 3.74 g/cm3

Melting point - 312.2 °C; 594.0 °F; 585.3 K

Boiling point - 465 °C; 869 °F; 738 K

Solubility in water - 20 g/L (25 °C)

Acidity (pKa) - 9.2

Chemical properties:

Formula - As_2O_3

Molar mass - 197.841 g/mol

Density - 3.74 g/cm³

Melting point - 312.2 °C

Boiling point - 465 °C

Medicinal uses:

In Chinese medicine, Arsenic is known as *Pi Shuang* and is still used to treat Cancer and other degenerative diseases. In Homeopathy, it is called *Arsenicum album*. [37]

Arsenic trioxide is a chemotherapeutic agent used to treat leukemia. It is assumed that Arsenic trioxide induces Cancer cells to undergo apoptosis. It is used as a cytostatic in the treatment of acute myeloid leukemia. The combination therapy of Arsenic trioxide and all-trans Retinoic acid (ATRA) has been approved by the U.S.A. Food and Drug Administration (FDA) for treatment of certain leukemias. University of Hong Kong developed a liquid form of Arsenic trioxide that can be administered orally.

Toxic effects:

Toxic effects are also well known upon inhalation or upon contact to skin. Symptoms of Arsenic poisoning are vomiting, abdominal pains, diarrhea sometimes with bleeding, convulsions, paralysis, cardiovascular problems, anaemia, inflammation of the liver and kidneys. These are followed by the appearance of characteristic white lines (Mees stripes) on the nails and by hair loss.

Chronic Arsenic poisoning is called as Arsenicosis. It affects workers in smelters, in populations whose taking water contains high levels of Arsenic (0.3–0.4 ppm), and in patients treated for long time with Arsenic-based medicines[38].

SIDDHA ASPECT OF*KUPPAIMENI (Acalypha indica):*

Other names:

Arimanjari, Poonai vananki, Meni

Vernacular names:

English name – Indian Acalypha, cat's struggle

Sanskrit name – *Arittamanjarie*

Telugu name – *Kuppi-chette*

Malayala name – *Kuppamani*

Kannada name – K*uppigida*

Parts used – Leaf, root, whole plant.

Taste – Bitter, Pungent, Potency – Hot, Division – Pungent

Habitat:

A small shrub found all over India in gardens and roadsides. Its height is about 1-2 feet.

General properties:

"தந்தமூ லப்பிணிதீத் தந்திடுபுண் சர்வவிடம்

உந்துகுன்மம் வாதம் உதிரமூ - லந்தினவு

சூலஞ்சு வாசம் தொடர்பீ சங்கபம்போம்

ஞாலங்கொள் மேனியத னால்".

-பதார்த்த குண சிந்தாமணி.

It is used in the treatment of tooth canal diseases, burns, stomach pain, *vaatha* diseases, piles, itching, asthma, rhinorrhea and cough.

Action:

- ❖ Anthelmintic
- ❖ Anodyne
- ❖ Cathartic
- ❖ Diuretic
- ❖ Expectorant [39A]

BOTANICAL ASPECT OF *KUPPAIMENI (Acalypha indica):*

Taxonomical classification:

Kingdom - Plantae

Order - Malpighiales

Family - Euphorbiaceae

Genus - Acalypha

Species - A. indica

Binomial name - *Acalypha indica* L

Habitat:

It is an annual herb and about 75 cm in height. Its leaves are 3-8cm long, ovate, thin usually 3-nerve, flowers auxiliary erect spikes, female flower supported by conspicuous wedge-shaped bracts, male flower: minute.

Distribution:

This species is globally distributed from Tropical Africa to Malaysia. Within India, it occurs throughout the plains.

Vernacular names:

English name - Indian Acalypha, three-seeded Mercury, Indian nettle

Ayurvedic name - *Kuppi, Haritamanjari, Muktavarchaa*

Pharmacognacy:

Whole plant contains alkaloid 'Acalyphin'. Its root contains a Cyanogenetic glucoside and Triacetonamine. This glucoside crystallises in two forms, thin hexagonal plants (m.p.,182-4^{o}) and fine silky needles (m.p.,108^{o}). Stigmasterol isolated from roots and leaves; Acalyphol acetate isolated only from leaves.

Action:

Leaf - Antibacterial

Whole Plant - emetic, expectorant. [40]

Medicinal uses:

Leaves used in the treatment of scabies, bronchitis, asthma, pneumonia. Tincture of fresh plant is used in Homeopathy for incipient phthisis with bloody expectorations, emaciation and arterial haemorrhage.[41]

SIDDHA ASPECT OF *VETRILAI(Piper betle):*

Other names:

Thaampoolam, thaampoolavalli, thiraiyal, nagavalli, mellilai, vellilai, melladagu

Vernacular names:

English name - Betal leaf

Sanskrit name - *Nagavalli*

Telugu name - *Tamalapaku*

Kannada name - *Vilayadele*

Malayala name - *Vetilla*

Habitat:

It is a climber, cultivated in tropical places.

Parts used – Leaves; Taste –Pungent; Potency – hot; Division – Pungent

General properties:

"ஐயம் அறுங்காண் அதன்சாரங் கொண்டக்காற்

பையச் சயித்தியம்போம் பைந்தொடியே - மெய்யின்

கடியின்குணம்போகுங்காரவெற்றிலைக்குப்

படியுமுத் தோடமிதைப் பார்".

-பதார்த்த குண சிந்தாமணி.

It is effectivein the treatment of *kapha* diseases, urticaria, diseases of *Tridosa*, cough and hoarseness of voice.

Actions:

- Stomachic
- Antiseptic
- Aphrodisiac
- Astringent
- Carminative
- Stimulant
- Sialogogue
- Galactogogue
- Febrifuge [39B]

BOTANICAL ASPECTOF *VETRILAI (Piper betle):*

Taxonomical classification:

Kingdom - Plantae

Order - Piperales

Family - Piperaceae

Genus - Piper

Species - P. betle

Binomial name - *Piper betle* L

Habitat and distribution:

Betal plant is a very popular plant in Indonesia and originated from South and South East Asia. These plants grow vines or leaning on another tree. The length of the Betel leaf is about 5-8 cm wide and 2-5 cm and bright green in colour.

Vernacular names:

English - Betel pepper.

Ayurvedic - *Taambula, Naagvallari, Taambulvalli, Saptashiraa*

Unani - *Paan, Tambool*

Nutritional composition:

Water - 85-90%, Carbohydrate - 0.5-6.10%, Protein - 3-3.5%, Fat - 0.4-1.0%, Minerals - 2.3-3.3%, Fibre - 2.3%, Vitamin A - 1.9-2.9 mg/100g, Vitamin C - 0.005-0.01%, Chlorophyll - 0.01-0.25%, Nicotinic acid - 0.63-0.89 mg/100g, Calcium - 0.2-0.5%, Potassium - 1.1-4.6%, Thiamine - 10-70µ g/100g, Phosphorus - 0.05-0.6%, Iodine - 3.4 µ g/100g, Riboflavin - 1.9-30µg/100g, Nitrogen - 2.0-7.0%, Tannin - 0.1-1.3%, Iron - 0.005-0.007%.

Phytochemicals:

Chavibetol (53.1%), Chavibetol Acetate (15.5%), Caryophyllene (3.79%), Allyl Pyrocatechol Diacetate (0.71%), Camphene (0.48%), Chavibetol Methyl Ether (0.48%), Eugenol (0.32%), α-Pinene (0.21%), α-limonene (0.14%), Safrole (0.11%), 1,8-cineole (0.04%), p-cymene (0.11%) and Allylpyrocatechol Monoacetate determined in leaf oil.

Action:

Leaf — stimulant, astringent, antiseptic

Essential oil from leaves — antispasmodic, antiseptic

Fruit — bechic [42]

Medicinal properties:

Administration of the leaf extract resulted in decreased tumour burden and tumour incidence and a delay in the onset of mammary tumour in Wister rats. The alcoholic extract of the leaf is found to show antispermatogenic and antiandrogenic effect in animal study. In Ayurveda, betel is used to cure worms. Chewing areca nut and betel leaf is a remedy for bad breath. [43]

SIDDHA ASPECT OF *PARUTHI (Gossypium herbaceum):*

Other names:

Aachatha nabalai, pari, uthuri, kaarpasam, pannal

Vernacular names:

English name – Indian cotton plant, Common cotton

Sanskrit name – *Karpasa*

Telugu name – *Paratti*

Kannada name – *Hatti*

Hindi name – *Kapas*

Malayala name – *Parithi*

Habitat:

It is an annual shrub and is cultivated in India and Srilanka. There are two varieties of cotton plant, red cotton and white cotton, red cotton is used for medicine.

Parts used – Leaf, flower, seed, bark, root bark

Taste – Astringent, sweet; Potency – Hot; Division- Pungent

General properties:

"பருத்தியிலை மொக்கிரண்டைப் பாலிலரைத் துண்ண

வருத்துகின்ற மேகமெல்லாம் மாறும் - பருத்த

விரத்தபித்தத் தோடு விரணவீக் கம்போம்

அரத்தவிதழ் மாதே யறை".

-பதார்த்த குண சிந்தாமணி.

It is used in the treatment of leucorrhoea, hypertension, ulcer, swelling, piles, dysentery, chronic wound and epistaxis.

Actions:

- Astringent
- Tonic [39C]

BOTANICAL ASPECT OF *PARUTHI* (*Gossypium herbaceum*):

Taxonomical classification:

Kingdom - Plantae

Order - Malvales

Family - Malvaceae

Genus - Gossypium

Species - *G. herbaceum*

Binomial name - *Gossypium herbaceum* L

Habitat and distribution:

It is a small shrub, cultivated mainly in Maharashtra, Tamil Nadu, Andhra Pradesh and Karnataka.

Vernacular names:

English name - Asiatic Cotton, Levant Cotton, Uppam Cotton

Ayurvedic name - *Kaarpaasa, Kaarpaasi, Kaarpaasaka, Kaarpaasa*

Unani name - *Pambahdaanaa*

Siddha name - *Paruthi*

Phytochemicals:

The seed contains 21.3- 25.2% protein and 0.82-1.96% free Gossypol. The germ of the plant contains both Betaine and Choline. The flowers yield a glucosidal pigment, Gossypetin.

Action:

Root Bark - Diuretic, Oxytocic

Bark - Emmenagogue, Haemostatic.

Seed - Demulcent, Laxative, Expectorant, Abortifacient [44]

Medicinal properties:

Cotton seed is used to treat dysmenorrhoea and metrorrhagia and also used to expel placenta after birth and to increase lactation. It is used for gastrointestinal complaints such as

hemorrhages, nausea, and diarrhoea, as well as fevers and headaches. Cotton seed extract also has a potential use as a male contraceptive. In lab rat studies, it has been proved to stop early pregnancies. The Ayurvedic Pharmacopoeia of India recommends the seed oil for toning up the breast.

Ethyl ether and ethanol extracts of *Gossypium herbaceum* significantly decreased the blood glucose level. It not only lowers TC, TGL, LDL, VLDL levels but also increases the level of HDL, Therefore, it has potential role to prevent formation of atherosclerosis and coronary heart disease. [45]

SIDDHA ASPECT OF *VELLARUGU (Enicostemma axillare):*

Other name - *Vallari*

Habitat:

It is a shrub and growing in tropical countries.

Parts used – whole plant; Taste – Bitter; Potency – Hot; Division– Pungent.

General properties:

"குன்மமமொடு வாய்வு குடல்வாதம் சூலையிவை

சென்மம்விட் டோடிச் சிதையுங்காண் - வன்முலையாய்

உள்ளுறுகி ரந்திசொறி யொட்டிய சிறங்குமறும்

வெள்ளறுகு தன்னை விரும்பு".

It is efficient in the treatment of ulcer, intestinal diseases, throbbing pain, *vaatha* diseases, joint pain, abscess, itching, scabies, and leucorrhoea and skin diseases.

Actions:

- Tonic
- Stomachic
- Alternative
- Laxative
- Febrifuge [39D]

BOTANICAL ASPECT OF *VELLARUGU (Enicostemma axillare):*

Taxonomical classification:

Family - Gentianaceae (Gentian family)

Genus - Enicostema

Botanical name - *Enicostema axillare*

Synonyms:

Enicostemma littorale auct non-Bl, *E. hyssopifolium* (Willd), *E. axillare* (Lam.) Raynal

Habitat and Distribution:

Indian Whitehead is a perennial herb growing up to 40 cm tall. Leaves are narrow-oblong, lance shaped. White flowers are borne in dense clusters in leaf axils. It is globally distributed in West Indies, India and Sri Lanka. It is distributed almost all over India up to an altitude of about 450 m.

Vernacular names:

English name - Indian Gentian

Ayurvedic name - *Naagjhvaa, Maamajjaka, Naahi, Tikshnapatra*

Unani name - *Naai, Naahi*

Siddha/*Tamil* - *Vellargu*.

Pharmacognosy:

Whole plant contain alkaloids; Gentianine, Erythrocentaurin, Enicoflavine and Gentiocrucine; Flavonoids—Apigenin, Genkwanin Iso-vitaxin, Swertisin, Saponarin and O-Glucoside derivatives of Sylwertisin and Isoswertisin; glucosides—Swertiamarin, Triterpene betulin.

Action:

- Bitter
- Tonic

- Stimulant
- Blood Purifier
- Anti-rheumatic
- Anti-Inflammatory
- Anthelmintic
- Cardiostimulant [46]

Medicinal properties:

The whole plant of Indian Whitehead is helpful in the treatment of diabetes, joint pain, rheumatism, abdominal ulcers, dermatitis, swelling, malaria and insect poisoning.

This plant is used as a substitute for *Swertia chirayita*, and it is found to be effective against malaria. It contains Ophelic acid which is also present in *Chiretta*. The root extract showed antimalarial activity both in vitro and in vivo[47].

SIDDHA ASPECT OF THULASI (Ocimum sanctum):

Other names:

Ari, Ramathulasi, Krishna thulasi, Thiruthulai, Thulavu, Kullai, Vanam, Viruntham, Thuzhai, Maalalangal

Vernacular names:

English names - Holy basil, sacred basil

Sanskrit name – *Sami*

Telugu name – *Thulasi*

Hindi name – *Sami*

Malayala name – *Thulasi*

Habitat:

It is an aromatic shrub growing throughout the country. It grows up to 2-3 feet height, there are several varieties in *Thulasi* like *Nalthulasi, Karunthulasi, Naai thulasi, Nila thulasi, Kal thulasi and* Mul *thulasi.*

Parts used – leaf, seed.

Taste - Pungent; Potency – Hot; Division – Pungent

General properties:

"போலாட்டி வித்துதிரப் புண்பீனி சப்பிணியை

மேலாட்டி வித்து விளங்குமே - சால

வருந்து முடற்குவெப்பு வாராமெ லோட்டும்

பிருந்தமைப்ப யித்தியத்தைப் பேய்".

It is used in the treatment of sinusitis, psychiatric disorder, headache, rhinorrhoea, sneezing, fever and *kapha* diseases.

Actions:

- Expectorant
- Stimulant
- Diaphoretic [39E]

MODERN ASPECT OF *THULASI (Ocimum sanctum):*

Taxonomical classification:

Kingdom - Plantae

Order - Lamiales

Family - Lamiaceae

Genus - Ocimum

Species - O. tenuiflorum

Binomial name - *Ocimum tenuiflorum or Ocimum sanctum* L.

Habitat and distribution:

Ocimum sanctum is an aromatic plant which is native to the Indian Subcontinent and widespread as a cultivated plant throughout the Southeast Asian tropics. It is an erect, much branched subshrub, 30–60 cm tall with hairy stems and simple opposite green or purple leaves that are strongly scented. The flowers are purplish in elongate racemes in close whorls. The two main morphotypes cultivated in India and Nepal are green-leaved (*Sri or Lakshmi Tulasi*) and purple-leaved (*Krishna Tulasi*)

Vernacular names:

English names - Holy Basil, Sacred Basil

Ayurvedic names - *Tulasi, Surasaa, Surasa, Bhuutaghni, Suravalli, Bahumanjari, Deva dundubhi, Apet-raakshasi*

Unani name - *Tulasi*

Phytochemistry:

Leaves and flowers contain 0.075 and 0.095% of volatile oil respectively. Major components of the essential oil are Eugenol, Carvacrol, Nerol and Eugenolmethylether. Leaves have been reported to contain Ursolic acid, Apigenin, Luteolin, Apigenin-7-O-Glucuronide, Luteolin-7-O-glucuronide, Orientin and Molludistin. β-Caryophyllne, Eugenol and Humulene are detected in flower oil. Leaves afforded β-carotene and Ursolic acid; determination of Caryophyllene, Bornyl acetate and Eugenol in essential oil.

Nutrition value:

Contains vitamin C and A, and minerals like [9] Calcium, Zinc and Iron, as well as chlorophyll and many other phytonutrients. Also enhances the efficient digestion, absorption and use of nutrients from food and other herbs. Protein: 30 Kcal, 4.2 g; Fat: 0.5 g; Carbohydrate 2.3 g; Calcium: 25 mg; Phosphorus 287 mg; Iron: 15.1 mg and edible portion 25 mg vitamin C per 100 g.

Anticancer effect of *Ocimum*:

Fresh leaf paste (topically) aqueous and ethanolic extract (orally) for their chemopreventive activity against 7, 12-dimethylbenzaanthracene (DMBA) induced (0.5%) hamster buccal pouch carcinogenesis. Incidence of papillomas and squamous cell carcinomas were

significantly reduced and increased the survival rate in the topically applied leaf paste and orally administered extracts to animals. Histopathological observation made on the mucosa confirmed the profound effect of the orally administered aqueous extract than other.

Action:

Leaf - carminative, stomachic, antispasmodic, antiasthmatic

Seed - used in genitourinary diseases

Root - antimalarial

Plant - adaptogenic, antistress

Essential oil - antibacterial, antifungal [48]

Medicinal uses:

The leaf of this plant used in the treatment of rhinitis, asthma, bronchitis, ulcers, liver diseases and influenza; the seed in psychological disorders, including fear-psychosis and obsessions[49].

3.3. PHARMACEUTICAL REVIEW:

Chenduram:

Definition:

Chenduram is a category of medicines made from metals or minerals (Arsenicals or Mercurials or salts) by grinding them with specified juices or distillates or extracts and subjecting them to a process of sublimation or calcination or burning or frying or exposing to insolation till the characteristic reddening of the product takes place. The *Chendooram* are said to retain their potency for 75 years.

Method of preparation:

Usually two methods of preparation are adopted in their processing though there are some exceptions and variants.

1. Sublimation by the sand – bath process

2. Calcination.

1. Sublimation by the sand - bath process *(Kuppi Erippu)*:

If the *chendooram* has Sulphur and Mercury as its components, Sulphur is ground to a fine powder in the mortar and grinding should be continued with the addition of the given quantity of Mercury, till a black impalpable mobile powder is obtained. Only after this, the other ingredients are to be added.

In the conventional set up of the sand –bath sublimation contrivance, a heat resistant glass flask with a long neck is used as the container for the drug ingredients. Ceramic ware had also been in use. Before being put to use, these container are wound around with clay smeared cloth ribbons so as to give seven superimposed layers, leaving open the mouth of the flask. The flask thus encased should be kept for perfect drying of the covering.

It has been found in recent times that one could make use of the enameled Iron bowls instead of glass flasks.

When using enameled Iron bowls, two identical bowls of appropriate dimensions and capacity should be selected and checked for neat contact of rims when juxtaposed. Then small holes should be punched along the margins so that the two bowls could be fastened with a bonding wire (metallic). Then a perforation is made in the centre of the bottom of one of the bowls. Having prepared the bowls thus, they should be secured and bound by pasting the binding wire through the marginal holes. This would produce a capsule with a top orifice. Clay smeared cloth tape is wound around as would be done for the glass flask, leaving the central opening uncovered. This opening is the one through which the reaction going on inside is inspected by inserting a probe.

The sand – bath is set up by taking a wide earthern trough and spreading fine gravel or coarse sand at the bottom to a depth of two centimetres.

The capsule into which the drug ingredients are put is placed on the gravel or sand and is properly centered. Then the sides packed with sands, leaving the top two centimetres unpacked and exposing the capsule. When using glass flasks, the neck should be just out of the sand. This setup is placed on the oven and heat is applied, by burning fire wood.

In the application of heat, there gradations are recognized. These three stages, mild, moderate and intense are best understood and mastered with some experience.

It is said that, if the flames are convergent and resemble a single tongue of flame as in a lamp, it is mild fire (*Deepakkini*). If several such tongues of flame lick the vessel and diverge like the flower of lotus, it is moderate (*Kamalakkini*). If the multiple tongues of flame fill the oven and enrich the sand bath. It is the intense stage of fire (*Katakkini*).

These stages of fire should be manipulated and followed as prescribed in the method of preparation. In general, the heating is spread over three continuous days. In such cases, mild, moderate and intense stages are maintained for 24 hours each, in that order of succession.

According to the composition and amount of Sulphur in the preparation, the mixture of drugs placed in the capsule will start melting sooner or later. Sulphur starts escaping first in the form of yellow vapour through the opening. Later it will start burning sending out a jet of blue flame. Just when the blue flame goes out if a long probe of steel wire is inserted into the orifice and drawn out the portion that enters the container will show a whitish coating. If the Sulphur is still present and not totally burnt out, the probe will have a black sticky coat, when there is no blackening of the probe and when whitish coat indicating should be closed and heating continued for one or two hours and then the heat withdrawn and the setup is allowed to cool by itself.

When the setup has cooled down, the capsule containing the medicine is taken out and the clay tape winding cut out. The material that has sublimed in upper bowl is gently tapped with suitable beater or lifted with a spatula. The sublimate collected should be finely ground in a mortar.

If the glass flasks had been used, the flask is carefully broken, open to collect the medicine that has sublimed in around the neck.

2. Calcination *(Putam)*:

The powder is ground in a *Kalvam* with specified fluids for a specified time. The paste is made into small discs and dried. They are put in earthern saucers (*man agal*) covered with another and the edge well sealed with mud cloth. It is allowed to dry. The cups are placed in the middle of cow – dung cakes and burnt. For *Putams,* generally pits of various depths and circumferences are made in the ground. Half of the pit is covered with cow – dung cakes. The earthern cups are placed and it is covered again with cow-dung cakes. The fire is put in the middle of the heap on all the four sides so that there would be uniform heat from all the sides.

All the metals and other ingredients are taken after the usual purification. In specified cases, specific purification (*Suddhi*) is mentioned; otherwise, it is to be taken as general method of purification for the drug as mentioned in Materia- Medica books.

Other methods of preparations:

1. Prepared without heating *(Araippu Chendooram)*
2. Prepared by open heating *(Erippu or Varuppu Chendooram)*
3. Prepared by applying heat in the range close to 100◦c *(Lagu Puda Chendooram)* [50].

3.3PHARMACOLOGICAL REVIEW

SIDDHA ASPECT OF THE DISEASE:

Hippocrates (ca. 460 BC – ca. 370 BC) described several kinds of Cancer, referring to them with the Greek word *Karkinos* (Crab or Crayfish). This name comes from the appearance of the cut surface of a solid malignant tumour, with "the veins stretched on all sides as the animal the crab has its feet, whence it derives its name"[51]. In Siddha system of medicine, Cancer is referred to *Vippuruthi* or *Putru.*

“புற்று நோயை மௌனப்பகைவன்

மறைந்திருந்து கொல்லும் பகைவன்”.

-திருமந்திரம்

Generally, a chronic tumour or swelling or ulcer is first identification of *Putru* in *Siddha* system. Tumours grow gradually and finally look like a *Putru*or cauliflower.

Causes:

- ❖ Taking excessive amount of salt and pungent.
- ❖ Taking large quantity of fish and meat.
- ❖ Making sleep in day time.

Types of *Vippuruthi*:

Vippuruthi is classified into seven types,

1. Karppa Vippuruthi

2. Kuvalai Vippuruthi

3. Vatha Vippuruthi

4. Pitha Vippuruthi

5. Seththuma Vippuruthi

6. Santhu Vippuruthi

7. Oodu Vippuruthi

Karppa Vippuruthi:

- Gastric regurgitation
- pain in side and lower abdomen
- dryness of skin
- lower abdominal swelling like pregnancy
- head ache

Kuvalai Vippuruthi:

- Pain in lower back, anal region and side of the chest
- Fever with shivering
- Cough with expectoration
- Abdominal pain and swelling

Vatha Vippuruthi:

- Pain and swelling in the lower abdomen, this swelling looks like a frog
- Fever
- Wound in the abdomen
- Pus discharge from the wound and abdominal distension

Pitha Vippuruthi:

- Hematemesis
- Paleness of the skin
- Burning sensation all over the body
- Shivering
- Mental disorder
- Hiccup

- Tastelessness of the tongue
- Fever
- Dehydration
- Hematoma and abdominal pain

Sethuma Vippuruthi:

Small tumour and abscess in the abdomen, abdominal pain, fever, cough and swelling of the body.

Santhu Vippuruthi:

Swelling in the side of the abdomen, this swelling is characterized by

- Shining
- Hardness
- cool and itching

Oodu Vippuruthi:

- Fever
- Blackish discolouration of skin
- Abdominal pain
- Giddiness
- Vomiting
- Diarrhoea and body pain.

Curable type of *Vippuruthi:*

1. Karpa Vippuruthi

2. Kuvalai Vippuruthi

3. Pitha Vippuruthi

4. Oodu Vippuruthi

Incurable type of *Vippuruthi:*

1. Santhu Vippuruthi

2. Sethuma Vippuruthi

3. Vatha Vippuruthi. [52]

3.2.2. MODERN ASPECT OF THE DISEASE:

Cancer:

Cancer known medically as malignant neoplasia, is a broad group of diseases involving unregulated cell growth. In Cancer, cells divide and grow uncontrollably, forming malignant tumours, which may invade nearby parts of the body. The Cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream. Not all tumours are Cancerous; benign tumours do not invade neighbouring tissues and do not spread throughout the body. There are over 200 different known Cancers that affect humans.

History:

Cancer has existed for all of human history. The earliest written record regarding Cancer is from circa 1600 BC in the Egyptian Edwin Smith Papyrus and describes Cancer of the breast. Hippocrates (ca. 460 BC – ca. 370 BC) described several kinds of Cancer, referring to them with the Greek word *Karkinos* (Crab or Crayfish). This name comes from the appearance of the cut surface of a solid malignant tumour, with "the veins stretched on all sides as the animal the crab has its feet, whence it derives its name." Galen stated that "Cancer of the breast is so called because of the fancied resemblance to a crab given by the lateral prolongations of the tumour and the adjacent distended veins". Celsus (ca. 25 BC – 50 AD) translated *Karkinos* into the Latin Cancer, also meaning crab and recommended surgery as treatment. Galen (2nd century AD) disagreed with the use of surgery and recommended purgatives instead. These recommendations largely stood for 1000 years.

In the 15th, 16th and 17th centuries, it became acceptable for doctors to dissect bodies to discover the cause of death. The German professor Wilhelm Fabry believed that breast Cancer was caused by a milk clot in a mammary duct. The Dutch professor Francois de la Boe Sylvius, a follower of Descartes, believed that all disease was the outcome of chemical processes, and that acidic lymph fluid was the cause of Cancer. His contemporary Nicolaes Tulp believed that Cancer was a poison that slowly spreads, and concluded that it was contagious.

The physician John Hill described tobacco snuff as the cause of nose Cancer in 1761. This was followed by the report in 1775 by British surgeon Percivall Pott that Cancer of the

scrotum was a common disease among chimney sweeps. With the widespread use of the microscope in the 18th century, it was discovered that the 'Cancer poison' spread from the primary tumor through the lymph nodes to other sites ("metastasis"). This view of the disease was first formulated by the English surgeon Campbell De Morgan between 1871 and 1874[53].

Epidemiology of Cancer:

Nearly seven lakh Indians die of Cancer every year, while over 10 lakh are newly diagnosed with some form of the disease. According to the latest World Cancer Report from the World Health Organisation (WHO), more women in India are being newly diagnosed with Cancer annually. As against 4.77 lakh men, 5.37 lakh women were diagnosed with Cancer in India in 2012.

In terms of Cancer deaths, the mortality rate among men and women in India is almost the same. While 3.56 lakh men died of Cancer in 2012 in India, the corresponding number for women was 3.26 lakh. One in every 10 Indians runs the risk of getting Cancer before 75 years of age.

Cancer of lip and oral cavity has emerged as the deadliest among Indian men while for women, it is breast Cancer. The top five Cancers in men are lip/oral cavity, lung, stomach, colorectum and pharynx, while among women they are breast, cervix, colorectum, ovary and lip/oral cavity.

The global Cancer burden jumped to 14.1 million new cases in 2012, with WHO saying the marked increase in breast Cancers must be addressed. The International Agency for Research on Cancer (IARC) 2012 estimated 14.1 million new Cancer cases and 8.2 million Cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008.

The most commonly diagnosed Cancers worldwide were those of the lung (1.8 million, 13% of the total), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%). The most common causes of Cancer death were Cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%).

Projections based on IARC 2012 estimates predict a substantive increase to 19.3 million new Cancer cases per year by 2025, due to growth and ageing of the global population. More

than half of all Cancers (56.8%) and Cancer deaths (64.9%) in 2012 occurred in less developed regions of the world, and these proportions will increase further by 2025[54] .

Causes:

Cancers are primarily an environmental disease with 90–95% of cases attributed to environmental factors and 5–10% due to genetics. Environmental, as used by Cancer researchers, means any cause that is not inherited genetically, such as lifestyle, economic and behavioural factors, and not merely pollution. Common environmental factors that contribute to Cancer death include tobacco (25–30%), diet and obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity, and environmental pollutants.

It is nearly impossible to prove what caused a Cancer in any individual, because most Cancers have multiple possible causes. For example, if a person who uses tobacco heavily develops lung Cancer, then it was probably caused by the tobacco use, but since everyone has a small chance of developing lung Cancer as a result of air pollution or radiation, then there is a small chance that the Cancer developed because of air pollution or radiation[55].

Classification of Cancer:

There are five broad groups that are used to classify Cancer.

1. Carcinomas are characterized by cells that cover internal and external parts of the body such as lung, breast, and colon Cancer.

2. Sarcomas are characterized by cells that are located in bone, cartilage, fat, connective tissue, muscle, and other supportive tissues.

3. Lymphomas are Cancers that begin in the lymph nodes and immune system tissues.

4. Leukemias are Cancers that begin in the bone marrow and often accumulate in the bloodstream.

5. Adenomas are Cancers that arise in the thyroid, the pituitary gland, the adrenal gland, and other glandular tissues [56].

Metastasis:

Metastasis is the spread of Cancer to other locations in the body. The new tumours are called metastatic tumours, while the original is called the primary tumour. Almost all Cancers can metastasize. Most Cancer deaths are due to Cancer that has spread from its primary site to other organs[57].

Head and neck Cancer:

Tumours of the head and neck are the sixth most common malignancy in the world, with a yearly incidence of more than 500,000 cases, and it comprises approximately 4% to 5% of all new Cancers and 2% of all Cancer deaths (100,000 per year). Most patients are older than 50 years, and incidence increases with age; the male-to-female ratio is 2.5:1. Approximately 34% of oral and pharyngeal Cancers present as localized disease, 46% present as loco regional (i.e., locally advanced or involving regional lymph nodes) disease, and 10% present as metastatic disease. Ninety percent of these Cancers involve squamous cell histology. The most common sites are the oral cavity, pharynx, larynx, and hypopharynx. Nasal cavity and paranasal sinus Cancers, salivary gland malignancies, and various sarcomas, lymphomas, and melanoma are less common.

Site-specific head and neck tumours:

Oral cavity:

The oral cavity includes the lip, anterior two thirds of the tongue, floor of the mouth, buccal mucosa, gingiva, hard palate, and retromolar trigone. Squamous cell carcinoma is the histologic type observed in most cases.

Oropharynx:

The oropharynx includes the base of the tongue, tonsils, posterior pharyngeal wall, and the soft palate.

Larynx:

Risk factors are a history of tobacco and/or alcohol intake. In addition, certain dietary factors and exposure to wood dust, Nitrogen mustard, asbestos, and nickel have been implicated as etiologic factors. The male-to-female ratio for laryngeal Cancer is 4.5:1, with a

peak incidence in the sixth decade of life. More than 95% of laryngeal Cancers are squamous cell Carcinomas.

Laryngeal Cancers can be supraglottic, glottic, and/or subglottic. Early Cancers not requiring laryngectomy are usually treated with radiation. If lymph nodes are involved, neck dissection and/or neck radiation is indicated. Locally advanced resectable tumors may be treated with surgery and adjuvant radiation if loco regional risk factors are present. An alternative is the use of combined radiation and chemotherapy.

Hypopharynx:

Early Cancers not requiring laryngectomy can be treated with surgery or radiation. Locally advanced resectable tumours may be treated with surgery followed by radiation or sequential or concomitant chemoradiation. In these cases, surgery involves total laryngectomy and partial or total pharyngectomy and neck dissection.

Nasal Cavity and Paranasal Sinuses:

Most tumours are squamous cell carcinomas and are usually slow growing with low incidence of metastasis. Carcinomas of the nasal cavity and paranasal sinuses are usually detected in patients in advanced stages because of the relatively silent tumour location. Treatment follows the same general guidelines as those for oral Cancer.

Nasopharynx:

It is extremely rare in most parts of the world, with an incidence of less than 1 case per 100,000 population. However, it is endemic in certain areas, including North Africa, Southeast Asia, China, and the far northern hemisphere. EBV is strongly associated with nasopharyngeal Carcinoma. This association has been demonstrated by serologic studies and by the detection of the viral genome in tumour samples. Diet (salt-cured fish and meat) and genetic susceptibility are other probable risk factors; tobacco and alcohol are not risk factors, except in a minority of cases.

Salivary Gland Cancer:

Salivary gland Cancers make up about 3% of all head and neck Cancers diagnosed in the United States yearly. Tobacco and alcohol consumption are not risk factors, except possibly in women. Ionizing radiation and certain occupational exposures (e.g., in workers in rubber

and automotive industries, wood workers, and farm workers) have been associated with the development of salivary gland Cancer.

The salivary glands are classified as major (parotid, submandibular, and sublingual) and minor (distributed along upper aerodigestive tract, predominantly in the oral and nasal cavities and the paranasal sinuses). Most of the salivary gland Cancers arise from the parotid glands; sublingual and minor salivary gland Cancers are rare.

Most salivary gland tumours are benign, and the most common histology is pleomorphic adenoma, which is characterized by slow growth and few symptoms, and is most frequently seen in the parotid gland. The most common presentation of benign salivary gland tumours is asymptomatic swelling of the lip, the parotid, or the submandibular or the sublingual glands. Persistent pain or neurologic involvement (mucosal or tongue numbness and facial nerve weakness) suggests malignant disease.

Surgery is the mainstay of treatment for all localized stages of salivary gland tumours. Postoperative radiation is indicated for localized tumours of high-grade histology, that are large, with close or positive margins, and/or positive regional lymph nodes. Radiation is the primary treatment for unresectable tumours. The role of chemotherapy is limited to the management of locally recurrent, unresectable disease or distant metastatic disease. There is no established standard chemotherapy for salivary gland Cancer. Regimens employing Cisplatin, Carboplatin, Anthracyclines, Taxanes, Cyclophosphamide, and 5-FU result in transient responses in 14% to 30% [58].

Other head and neck tumours:

Sarcoma:

Soft tissue sarcomas of the head and neck are relatively rare. Of head and neck sarcomas, 80% are seen in adults and 20% are in children. These tumours are heterogeneous and can present in any head and neck site, commonly as a submucosal or subcutaneous painless mass. In the hypopharynx and nasopharynx, the presenting symptoms may be cranial nerve abnormalities or airway or swallowing difficulties. As in sarcomas at other sites, grade is an important prognostic indicator. High-grade, aggressive tumours such as malignant fibrous histiocytoma, angiosarcoma, osteogenic sarcoma, neurofibrosarcoma, and soft part sarcomas tend to be locally aggressive and spread along neurovascular structures, fascia, and bone. In addition to aggressive local behavior, there is a high risk for metastatic disease, particularly in

lung, bone, central nervous system, and liver. Metastatic disease may occur without local lymph node involvement. Sarcomas may arise after radiation therapy, but this is very uncommon in the head and neck region. The prognosis for these secondary sarcomas may be worse than for primary sarcomas.

Treatment depends on stage, age of the patient, tumour type, location, and size. Wide margin en bloc resection is the goal, but may not be possible because of the proximity of vital structures. Adjuvant postoperative radiation and/or brachytherapy can improve local control in aggressive sarcomas. The major indications for adjuvant radiation are high-grade sarcomas or positive margins, lesions greater than 5 cm, and recurrent sarcoma. Elective neck radiation is not necessary because the incidence of occult positive lymph nodes is low. Soft tissue and possibly osteogenic sarcomas may benefit from adjuvant or neoadjuvant chemoradiation. Such patients should be referred to clinical trials when possible. Overall survival rate approaches 60% for patients with sarcomas of the head and neck.

Melanoma:

Mucosal melanomas represent less than 1.5% of all melanomas. About 50% of mucosal melanomas occur in the head and neck, and more than 20% of melanomas that occur in the head and neck region are mucosal. The age of diagnosis is 60 to 80 years. The hard palate is the most common site. Nearly one-third of these tumours are amelanotic. The proportion of mucosal melanomas is higher in African American and Hispanic populations than in white populations. Although rare in the United States, mucosal melanomas are more frequent in Japan and in some parts of Africa. Mucosal melanomas may be multiple, may have satellite lesions, may invade angiolymphatics, and can metastasize. They behave more aggressively than skin melanomas. Lymph node metastasis is observed at presentation in up to 48% of patients. Surgery is the mainstay of treatment for local or locoregional disease. Prophylactic lymph node dissection is not recommended. Radiation, when used, is usually employed adjuvantly for positive margins or used palliatively for local recurrence or unresectability. Adjuvant use of radiation has not been shown to improve survival. Prophylactic nodal radiation is not recommended. Chemotherapy and Immunotherapy have been studied, but the effect of these interventions on survival when used as palliation or as adjuvant therapy has not been defined. Patients should be encouraged to enter clinical trials where available. Mean overall 5-year survival is 17%[59].

Oral Cancer:

Oral Cancer is one of the most common head and neck malignancies. Oral Cancer is a general term for oral cavity Cancers. It occurs in the majority, where squamous cell carcinoma, which is called the mucosa mutate. In clinical practice, oral Cancer, including Cancer gums , tongue , hard and softpalate, carcinoma of the mandible , oropharynx, lip Cancer and maxillary sinus. Cancer occurs in the facial skin and mucous membranes of oral cavity and so on.

Causes of Oral Cancer:

Long-term habit of tobacco, alcohol:

Most oral Cancer occurs in patients with long-term history of smoking and drinking.

Poor oral hygiene:

Poor oral hygiene, bacteria or fungi in the mouth breeding to create the conditions, thus contributing to the formation of Nitrosamines and their precursors. Coupled stomatitis, proliferation of some cells in this stage, more sensitive to carcinogens, are the variety of reasons which contribute to oral Cancer.

Long-term stimulation by foreign body:

Root or sharp cusp, inappropriate dentures long-term stimulation of oral mucosa, resulting in chronic ulcers and even Cancer.

Malnutrition:

Vitamin A deficiency can cause oral mucosal epithelial thickening, hyperkeratosis with the occurrence of oral Cancer. Demographic studies show that countries with low intake of vitamin A high incidence of oral Cancer. There are also inadequate intake of trace elements considered relevant, such as low Zinc content of foods. Zinc is indispensable for the growth of animal tissue elements, Zinc deficiency may lead to mucosal epithelial damage, and create favorable conditions for the occurrence of oral Cancer. Inadequate plant protein and animal protein intake may be associated with oral Cancer[60].

Leukoplakia and Erythema:

Oral Leukoplakia and hypertrophic Erythema often a precancerous lesion.

Associated lesions:

The relationship between oral Cancer and precancerous lesions:

White ulcers or blisters inside the buccal mucosa occurs, often occurs as pressure sores, poor sleep or eating habits (such as insufficient fruits), in general will heal within two weeks. If it is not cured in two weeks, must be examined to rule out the possibility of epithelial cell carcinoma.

Changes in the oral mucosa color:

Normal epithelium pink, red or a white color of polarization are not normal. If red with white, it is more serious situation, another example of the tongue appears dark red with white dot like, highly suspicious of Cancer.

Ulcer:

Over more than two weeks of oral mucosal ulcer has not yet healed.

Clinical manifestations:

- Lumps, nodules
- White, smooth scaly plaque appeared
- Red patches, ulcers, inflammation and other symptoms distinct can't be cured for a longer period
- Repeated oral bleeding for no apparent reason
- Numbness, burning, or dryness of mouth for no apparent reason
- Unusual difficulty in speaking or swallowing[61]

Differential diagnosis

Traumatic ulcers:

These ulcers often occur in the edges of tongue, the corresponding total area of the ulcers, or irregular teeth and root dental prosthesis, indicating that ulcers are caused by the stimulus. Ulcers are soft with no induration tend to heal in 1or2 weeks if the irritant is eliminated.

Tubercular ulcers:

These are almost secondary, mostly open tuberculosis direct result of the spread, often occurs in the soft palate, buccal mucosa and back of the tongue, shallow ulcers compared with Cancerous ulcer, ulcer base induration soft non-invasive, effective anti-TB treatment. Imaging and biopsy can accurately identify and diagnose.

Diagnosis:

When neck mass is the first presentation, the primary site can be located and biopsied in approximately 80% of cases. If no primary site is obvious, tissue diagnosis can be obtained by fine needle aspiration (FNA) biopsy of the node, with sensitivity and specificity approaching 99%. A non-diagnostic FNA does not rule out the presence of tumour.

Computerized tomography scan (CT scan) remains the primary imaging study for evaluation of metastatic adenopathy. Magnetic resonance imaging (MRI) may complement the CT scan. Positron emission tomography (PET) scans are being used more frequently to detect tumours that are not obvious on other scans.

Laryngoscopy and nasopharyngoscopy should be performed. With occult primary tumours, directed biopsies of the nasopharynx, tonsil, base of tongue, and pyriform sinus should be performed. Bilateral tonsillectomy will sometimes reveal the source of an occult Cancer. [62]

Treatment:

The management of patients with head and neck Cancer is complex. The choice of treatment modality depends on the stage and site of disease. Patients with locally advanced disease should be evaluated (prosthodontics, nutrition, speech, and swallowing) by a multidisciplinary team including otolaryngologist or head and neck surgical oncologist, radiation oncologist, medical oncologist, dentist, and personnel involved in rehabilitation before treatment is initiated.

In general, either surgery or radiation is effective as single-modality therapy for patients with early-stage disease (stage I or II) for most sites. The choice of modality depends on local expertise, patient preference, and functional result. For the 60% of patients with locally advanced disease (stage III, IV, and M0), combined-modality therapy is indicated. [63]

Surgery

The nature of the surgical procedure is determined primarily by the size of the tumour and the structures involved. Extensive surgeries and those involving function of the tongue. Frequently require muocutaneous flaps or microvascular free flaps to achieve a more functional reconstruction.

Resectability depends on the experience of the surgeon and the rehabilitation team. In general, a tumour is unresectable if the surgeon believes that all of the gross tumour cannot be removed or that local and distant control will not be achieved after surgery even with adjuvant radiation therapy. Generally, involvement of the skull base, pterygoid, and deep neck musculature, and of the major vessels portends a poor outcome with surgery as a primary modality.

Cervical lymph node dissections may be elective or therapeutic. Elective neck dissections are done at the time of surgery in patients with necks that are clinically negative when the risk of a positive lymph node is at least 30%. Therapeutic neck dissections are done for clinically obvious masses. This surgery is now rarely performed because of excessive morbidity, especially loss of shoulder function. The modified radical dissection preserves one or more of the non-lymphatic structures. In selective neck dissections, only certain levels of lymph nodes are removed on the basis of the specific lymphatic drainage from the primary site.

Radiation Therapy:

The use of radiation as a single therapy in early-stage tumours (i.e., T1 and T2) is as efficacious as surgery. The choice of therapy depends on expected quality of life, functional outcome, sequelae of therapy, and options for treatment in case of recurrence.

In locally advanced tumours (i.e., T3 and T4), radiation therapy is combined with surgery. In general, postoperative radiation is preferred over preoperative radiation according to the results of two randomized prospective studies that show superior local control and minimally increased survival in the postoperative radiation arm in hypopharyngeal Cancer patients. Postoperative radiotherapy is recommended for patients at high risk for local recurrence [i.e., T4 tumour, close or positive margins (<5 mm), perineural or perilymphatic or vascular invasion by the tumour, multiple or large positive nodes, and/or extracapsular invasion].

The radiation type varies for specific sites and for definitive versus adjuvant therapy. The standard fractionation regimen in the United States is 1.8 to 2.0 Gy once daily, 5 days per week. The total dose of irradiation for definitive treatment is in the range of 70 to 80 Gy depending on the treatment schedule given and on the ability to shield normal tissue.

Common severe acute radiation toxicity includes epidermitis, mucositis, loss of taste, xerostomia, dysphagia, and hair loss. Dental evaluation and necessary extractions should be performed before radiation because dental extractions in a radiated mandible can lead to osteonecrosis. Dentulous patients should be given prophylactic fluoride. Patients receiving radiation are at high risk for tooth decay due to the xerostomia caused by injury to the salivary glands as well as mucosal damage.

Brachytherapy can be used as a definitive treatment for early-stage tumours or combined with external beam radiation in more advanced lesions in selected tumours (e.g., tongue, floor of

mouth, tonsil, and nasopharynx) with excellent results. Brachytherapy is an option for recurrent Cancers of the head and neck, particularly in previously irradiated patients. [64]

Chemotherapy:

Until relatively recently, chemotherapy was used mainly for palliation of patients with locally recurrent or disseminated disease without proven survival advantage. Combination chemotherapy yields higher response rates but has increased toxicity and no proven survival advantage when compared with single agents. The choice of single-agent or combination chemotherapy depends on the patient's preference and performance status. Several Combination regimens have been developed to improve response rates. The combination of Cisplatin and infusional 5-Fluorouracil (5-FU) produces a 70% response rate and a 27% complete remission (CR) rate in chemotherapy-naive patients.

Platinum-based chemotherapeutic regimens and the single agent methotrexate are the most commonly used regimens for metastatic disease. Carboplatin may be slightly less active than Cisplatin for head and neck squamous Cancer, but Carboplatin combinations with other chemotherapy agents are generally better tolerated than those with Cisplatin. Carboplatin is preferred in patients at high risk for Cisplatin toxicity, that is, those with renal dysfunction, neuropathy, or hearing loss.

Both Docetaxel and Paclitaxel have shown antitumor activity. Several dosing schedules for Paclitaxel have been investigated. Three-hour infusions are probably the best balance between theoretically optimum exposure and tolerable toxicity. Docetaxel is usually administered at doses of 60 to 100 mg per m^2 every 3 to 4 weeks.

The role of chemotherapy has expanded significantly over the last decade because of the results of clinical trials incorporating chemotherapy in multimodality regimens for previously untreated disease.

Induction Chemotherapy:

Induction chemotherapy followed by definitive radiation therapy in patients responding to chemotherapy has been studied for organ preservation in patients with locally advanced Cancers of the larynx and of the hypopharynx. No significant survival difference has been demonstrated for chemotherapy followed by radiotherapy compared to surgery followed by radiotherapy in these patients. For laryngeal Cancer, concomitant Cisplatin and radiation

therapy leads to better local control. Presently, induction chemotherapy followed by radiation therapy can be considered standard only for patients with previously untreated locally advanced squamous Cancers in the hypopharynx.

Concomitant chemoradiation:

The rationale for concomitant chemoradiation is based on experimental evidence of synergism between chemotherapy and radiation that is theoretically mediated by interference of chemotherapy with multiple intracellular radiation-induced stress-response pathways involved in apoptosis, proliferation, and DNA repair. The finding that certain chemotherapeutic agents (e.g., Cisplatin, 5-FU, Taxanes, and Hydroxycarbamide) can induce radiosensitivity and increase log cell kill for radiation supports this treatment strategy. Cisplatin, the most extensively evaluated drug in recent large randomized trials, has the advantage of not having mucositis as toxicity, although as a radiation enhancer, it does increase radiation-induced mucositis.

Adjuvant Chemotherapy:

A large randomized study in resected patients with stage III or IV disease compared adjuvant radiation therapy with adjuvant chemotherapy followed by radiation. This trial showed improved local control and overall survival rates approaching statistical significance for a subset of patients treated with chemotherapy who were at high risk for local recurrence. Patients with low-risk disease did not benefit from adjuvant chemotherapy.

Adjuvant concomitant cisplatin and radiation in patients at high risk for recurrence after surgery has been studied both in Europe and in the United States. Both studies found a possible benefit in disease-free or overall survival for patients receiving concomitant cisplatin and radiation [65].

Prevention:

1. Avoid unnecessary prolonged illumination, prevent the emergence of lip Cancer
2. Avoid smoking and drinking
3. Patients wearing dentures
    a. Found that tissue under dentures pain, inflammation, should seek immediate medical attention. Strive to achieve early detection of Cancer, early diagnosis and early treatment, and insisted regularly checked.

4. Balanced diet
5. Do not drink and eat hot water and food, to avoid irritation of oral tissues
6. Unplug the tooth residual root and crown
7. Good wear dentures, does not stimulate tissue
8. Develop good oral hygiene habits
9. Regular brushing. Pay attention to nutritional balance, timely treatment

Four symptoms of oral Cancer to be alert:

If the mouth turns white, brown or black, it means a change in mucosal epithelial cells. Especially the oral mucosa becomes rough, thickened or showed induration, appeared oral leukoplakia , erythema, is likely to be Cancerous.

Unhealed ulcer:

Oral ulcers of the course is generally not more than two weeks, if the burning sensation, pain and other symptoms brought the matter still more than two weeks, to be alert to the possibility of oral Cancer.

Obvious pain:

Intially generally painless or only partial exception sense of friction, ulceration obvious pain, with further violations of the nerve tumor, can cause ear and throat pain.

Lymph nodes:

Multiple oral Cancer to nearby lymph node metastasis to the neck, and sometimes the primary lesion is small, and even the symptoms are not obvious, but they found a lymph node metastasis of Cancer cells. Therefore, such a sudden neck lymph nodes, need to check the mouth.

Dysfunction:

Zhang closed the tumour may infringe muscles and temporomandibular joint, resulting in the opening and closing movement is restricted.

Vaccines:

A number of infectious agents cause Cancer. Hepatitis B and C are linked to liver Cancer, some human papilloma virus (HPV) strains are linked to cervical and head and neck Cancer, and Helicobacter pylori is associated with gastric Cancer and lymphoma. Vaccines to protect against these agents may reduce the risk of their associated Cancers. The hepatitis B vaccine is effective in preventing hepatitis and hepatomas due to chronic hepatitis B

infection. Public health officials are encouraging widespread administration of the hepatitis B vaccine, especially in Asia, where the disease is epidemic. A four-valent HPV vaccine (Gardasil) is 100% effective at preventing infection. The vaccine is recommended for girls and women ages 9–26 years. Reduction in these HPV types could prevent >70% of the cervical Cancers worldwide[66].

3.6 PHARMACOLOGICAL REVIEW OF CANCER:

The pharmacological screening of plants, minerals and animals is an essential mean for the invention of new, harmless, and effective drugs. Over 50,000 plants have therapeutic virtues in the world, and around 80% of human use medicines based on plants and salts at least once in their life. Medicinal plants and mineral share diversified chemical constituents which are important for the discovery of new active molecules against many types of Cancer. Active compounds from many medicinal plants and minerals with effective cytotoxic properties were developed into AntiCancer drugs.

Nowadays it has become mandatory to monitor the quality of life of patients while in treatment of Cancer. There should be health awareness in the quality of life of Cancer patients treated with chemotherapeutic drugs are very much affected even long time after withdrawal of drugs. Therefore, the challenging task at this moment is to identify the quick and novel methods that can identify and develop molecules, which can be of therapeutic value in human Cancers. This urgently necessitates screening of a large number of compounds. For this purpose both, the *in vitro* and *in vitro* models are employed for systematic screening of an AntiCancer drug.

INVITRO METHODS:

Though animal models provide more predictable results, in vitro testing is still preferred prior to in vivo testing of a potential chemotherapeutic agent. There are following advantages of in vitro models over in vivo models.

1. These are less time consuming.
2. More cost effective.
3. Small quantities and large number of compounds can be tested.
4. These are easier to manage.

In addition, in vitro cultures can be cultivated under a controlled environment (pH, temperature, humidity, oxygen / Carbon dioxide balance, etc.) resulting in homogenous batches of cells and thus minimizing experimental errors.

The in *vitro* methods are not free from disadvantages also and they often furnish false positive results (compounds show no activity in vivo) and false negative results (compounds shows no activity in vitro but show activity in vivo as they need to be biotransformed in vivo to a pharmacologically active compound). A second pitfall is that the role of pharmacokinetics in determining drugs effect cannot be evaluated in vitro. In addition, geometry of solid tumors in vivo is very different from that of cells growing in vitro in suspension or monolayer cultures.

Ideal Characteristics of an In vitro Screening Method:

An ideal in vitro screening method should be simple, economical, reproducible, rapid and sensitive. The assay should be applicable to large number of tumor types and test compounds. The choice of the cell lines should be representative of clinical situation as close as possible. The range of drug concentrations used in vitro should be comparable to that expected for in *vivo* treatment. The assay should be able to process a large number of samples quickly and in an automated fashion. Data acquisition should be simple, easily interpreted and applied. At present no such system is available.

The goal of a screening assay is to test the ability of a compound to kill cells, at the same time, the assay should be able to discriminate between replicating cells and non replicating cells (quiescent cells that are dead or dying apoptosis). Different assays take disadvantage of various properties of cells as mentioned below.

Table: 1. Different assays take disadvantage of various properties of cells

S.No	Cell properties	Assay
1.	Enzymatic properties	Tetrazolium salt assay(MTT)
2.	Protein content/synthesis	Sulphorhodamine B assay
3.	DNA content/synthesis	H-Thymidine uptake Newer fluorescent analogues with flow cytometry
4.	Membrane integrity	Dye exclusion tests
5.	Clonogenic properties	Clonogenic assay

6.	Cell division	Cell counting assay

Tetrazolium Salt Assay (Microculture Tetrazolium Test or MTT):

MTT assay is an international accepted in vitro method for antiCancer drug screening. Though viable cells can be measured using several other staining procedures also but these procedures suffer from drawbacks that they require washing steps thereby increasing processing time and sample variation. The multiwell plate scanning spectrophotometers can quickly measure large number of samples with a high degree precision and accuracy. Ideally, a colorimetric assay for living cells utilize a colorless substrate that is modified to a coloured product by any living cells, but not by non-viable or dead cells or culture medium. However, MTT assay utilizes a color reaction as a measure of viable cells. The assay is dependent on the cellular reduction of 3-(4,5-dimethylthaizol-2-yl) -2,5-diphenylltetrazolium bromide, a tetrazolium salt to a blue formazan product by the mitochondrial dehydrogenase of viable cells/ metabolically active cells. The intensity of blue colored Formanzan produced is directly proportional to cell viability.

The cells from a particular cell line when in log phase of growth are trypsinized, counted in a hemocytometer and adjusted to appropriate density in a suitable medium and then inoculated in different multiwell plates (usually 96-well plates). The cells are treated with various concentrations (in replicates) of drugs for specified duration (usually 1 to 4 days), after which MTT dye is added in each well and plates are incubated at 37° for 4 h in a co_2 incubator. The plates are taken out of incubator and dark blue coloured Formazan crystals are thoroughly dissolved in isopropanol / DMSO at a room temperature. The plates are then read on an ELISA reader at 570 nm. The percent cell viability with respect to control is calculated using the formula:

$$\% \text{ Cell viability} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

This assay has been successfully used by us. The DMSO as a solvent rapidly solubilizes the serum as well as Formazan and use of spectrophotometric grade DMSO gives stable "background" absorbance levels. Other solvents like isopropanol, propanol, hexane and dimethylformamide though used , do not solubilize serum at concentrations exceeding 0.0625 percent.

The advantage of this assay is that it can be run on microtiter dishes on hundreds of cell samples at one time so that the various drug concentrations can be used to get an idea of the dose response relationship for each drug tested. As a result, this assay can be adopted for the determination of $IC_{50}$of drugs (concentration of drug required to inhabit 50% cell growth).

Further this assay is relatively simple and therefore easy to perform. It can be used for both adherent and suspension cell lines. This method is cheap, requires low number of cells, manageable and a large number of drugs can be quickly screened for antiproliferative activity. However, the assay suffers from the drawback of giving false results due to the inclusion of cells that might be metabolically active but not capable of dividing (nonreplicating). In addition, drugs whose mechanism of section might spare mitochondria may not yield positive results in this assay, especially for short incubation times. Also, use of DMSO warrants safe handling by laboratory personnel.

Sulphorhodamine B Assay:

The Sulphorhodamine B (SRB) assay measures whole-culture protein content, it should be proportional to the number of cell. Cell cultures are stained with a protein staining dye, Sulphorhodamine B. It is a bright pink dye, which binds to basic amino acids of cells. unbound dye is then removed by washing with acetic acid , and protein-bound dye extracted using unbuffered. Tris base for determination of optical density in a computer-interfaced, 96-well microtiter plate reader. Since dead cells either lyse or are lost during the procedure, the amount of SRB binding is propotional to the number of live cells left in a culture after drug exposure. This assay can be used to measure the cellular protein content of both adherent and suspension cultures. Screening capacity, reproducibility and quality control all appear to be enhanced in this assay relative to the tetrazolium salt assays. The assay is more cumbersome and time consuming compared to the MTT assay. Non-replicating and dead cells might contribute to the total protein and interfere with the results.

H-thymidine Update Assay:

In this assay tumor cell suspensions are exposed to the drug continuously for 5 days, after which a radio-labeled precursor (^{3}H-thymidine) is added during the final 48 hours of the assay to label proliferating cells. The replicating cells will incorporate [^{3}H] -thymidine into their DNA, which can then be determined either by autoradiography or by liquid scintillation counting, Autoradiographic determination of the [3H]-thymidine, though, is time- consuming but it provides information on tumor growth kinetics. This can generate DNA histograms, which can provide information on the ploidy status of the cells. This assay looks at cells, which have actively replicating DNA and hence are viable, Nonreplicating or dead cells will not be counted in this case. The assay can be used for both adherent and suspension cell lines. The assay is rapid, relatively inexpensive, and feasible in the majority of tumor types. However, it will not differentiate between malignant and non malignant cells and might lead to false-negative predictions if lethally damaged cells undergo a final division.

Fluorescence:

Fluorescent dyes may be used in conjunction with microscopic evaluation methods as an in vitro chemosensitivity assay. Cells are exposed to fluorescent-labeled precursors after drug-exposure. The replicating cells will incorporate labeled precursor into their DNA and the resulting fluorescence is then measured by flow cytometry. This assay also looks at actively replicating cells and hence dead or nonreplicating cells are not counted. In addition, the assay does not involve the use of radioactivity and is useful for adherent and suspension cell-lines. Also using flow cytometry it is possible to determine that in what phase of the cell cycle the cells are. The quantitation of apoptotic cells is also possible. However this, assay requires the data to be analyzed by an expensive and sophisticated fluorescence activated cell-sorter(FACS) instrument. Because of technical difficulties in applying flow cytometry to primary tumor specimens, data on the predictive value for clinical response for this assay are too scarce to permit definitive conclusions.

Dye Exclusion Tests:

Early attempts to use exclusion of vital dyes like trypan blue, eosin, or nigrosin to predict chemosensitivity were unsuccessful. These assays relied on the structural integrity of the cells .Dead cells would have lost membrane integrity and hence would take up vital dyes like trypan blue. This method was mainly used because of its technical simplicity No

prospective trials of these assays have yet been performed , however, to demonstrate their ability to predict for response or lack of response. This DISC assay is drug sensitive assay, which relies on structural integrity of the cells. In this assay, cells are incubated with drugs for 4 days. Dead cells are stained in suspension with fast green dye with or without nigrosin. The specimen is centrifuged and disks of cells are collected in the microscopic slides. Live cells are then stained with hematoxylin-eosin. Duck erythrocytes are used as control. The end point of the study is the morphologic identification of tumor-cell cytotoxicity compared with the internal control standard of duck erythrocytes. The DISC assay measures cell kill in both dividing and non-dividing and tumor cell population.

Clonogenic Assays:

A concern in the use of antiproliferative assays is that they measure growth inhibition rather than cell killing. This is particularly important for drugs that act by arresting cells at check points in the cells cycle. Checkpoint arrest is a survival response of cells that allows repair of DNA damage and is therefore not directly related to the induction of cell death. Thus, cells that act by arresting cells at checkpoints may show lower IC50 but increased survival. Clonogenic survival assays on the other hand, measure loss of tumor cell reproductive viability(the ability of a single cell to form colonies).It is the most direct method of measuring cytotoxic activity of a drug, In clonogenic assays single-cell suspension are prepared from tumor biopsies and exposed to antiCancer agents to be tested . Cells are then rinsed and plated in a semisolid medium (agar or methy1 cellulose),a medium that precludes proliferation of nonmalignant cells in the specimen.[15] After 14 to 28 days, some cells will have undergone several divisions and will have formed tumor colonies, which can be quantified in a visual or semiautomated fashion. Nonreplicatig and dead cells are not coated in this case. The number of colonies from the treated cells is compared with the number of colonies from the untreated control cells and the fraction of control growth provides an index of drug activity. Traditional clonogenic systems suffer from a number of significant technical problems like long incubation time(at least 14 days)before results can be made available to the clinician. The assay is labor-intensive, costly, and cannot be used for suspension cell-lines.

Cell Counting Assay:

Cells are cultured in the presence of drug for 2-5 culture-doubling times, after which the cell number is estimated using a hemocytometer or a cell counter. The assay is easy to

perform, rapid and can be used for both adherent and suspension cell lines. However, dead and nonreplicating cells can be counted in this assay by the cell counter. The IC_{50} values can be calculated in all the above assays.

***INVIVO* METHODS:**

In vivo models are advantageous over in vitro models in the sense that they detect host-mediated activity, are relatively predictable and estimate therapeutic ratio. However, as compared with in vitro systems, their sensitivity is low, are costly, time consuming and large number of samples cannot be handled and are difficult to manage. After all, animal models are used both toxicological studies and for detecting preclinical antiCancer efficacy. They are able to detect agents irrespective of their mechanism of action. The drugs with high degree of efficacy and broad spectrum of activity in animal models are usually expected to be effective in clinical Cancer, however, there are exceptions also which could be due to metabolic differences and heterogeneity of Cancer cells between human and rodents. Despite these differences animal models are widely used to support the results obtained from in vitro studies. The most promising candidate compound is tested in more than one animal model. Dose response relationship, combined effect of drugs, modes of their antiCancer action and organ specificity are established. Varied drug dosage forms, doses and animal strains and animals of a particular age group may be used. The selected animal models should be representative of high incidence of humans. The in vivo antiCancer drug screening methods are described under the following headings:

A. Chemically induced tumor models

B. Models involving cell line/tumor pieces implantation.

Chemically Induced Tumor Models:

Chemical carcinogens are well-known to account for about 80% of all Cancers and are used to induce Cancer in animal models. Carcinogens require metabolic activation before inducing carcinogenesis. The epidemiological studies indicate that human carcinogenesis occurs through multiple steps in the same way as in mouse skin. The concept of multistep carcinogenesis was first of all developed in rodent skin models in 1940s and applies to Cancers to many species and cell types. Experimental carcinogenesis involves following three steps:

- Initiation is due to exposure to carcinogens transforming the normal cell to a Cancer cell.
- promotion is due to the triggering of uncontrolled growth of the transformed cell.
- Malignant conversion is caused due to unlodging of Cancer cells from the original site its transportation by circulation and the establishment of secondary tumors in the body.

The exact sequence of cellular, biochemical and molecular genetic events may differ between tissues and species, the overall concept seems to be directly applicable to clinical Cancer and thus in future multistage mouse skin carcinogenesis model will be of immense utility for further understanding the mechanisms of epithelial carcinomas in human beings.The experiment is well designed; dose of the carcinogen as well as drug treatment schedule is standardized by conducting pilot studies. This helps in accurate evaluation of the test compound.

DMBA-induced Mouse Skin Papillomas:

This is a classical two-stage experimental carcinogenesis model. Mouse skin is generally most sensitive to epidermal carcinogenesis, Rats, hamsters and rabbits are less sensitive and guinea pig is very resistant 2. SENCAR mice are highly sensitive to DMBA-induced skin tumors .Swiss albino mice are relatively less susceptible to tumor induction. DMBA acts as an initiator and 12-o-tetradecanyl-phorbol-13-acetate(TPA) is used as a promotor to induce skin papillomas and squamous cell carcinomas. Mice are topically applied a single dose of 2.5µg of TPA in 0.2 ml acetone twice weekly on the same site starting one week after DMBA application. Papillomas begin to appear after 6 to 7 weeks of application of TPA. Weekly observations are made to monitor tumor development till the experiment terminates after 18 weeks. Percent tumor incidence and multiplicity of treatment group is compared with DMBA control group. Drug under test can be administrated either topically or by oral route. The tumor incidence in this model is usually about 100% in DMBA controls. In various laboratories, however, repeated topical application of DMBA alone has also been shown to induce carcinogenesis 3. The development of papillomas in DMBA treated Swiss albino mouse (24th week).

MNU-induced Tracheal Squamous Cell Carcinoma in Hamster:

In this model, 5% solution of MNU in the normal saline is administrated once a week for 15 weeks using specially designed catheter, which exposes a defined area of the trachea of male

Syrian Golden hamsters to the carcinogen. Fifteen weeks MNU administration produces tumors in 40-50% animals within 6 months. Test drug efficacy is measured as percentage reduction of tumor incidence compared with carcinogen control.

DMBA-induced Oral Cancer in Hamster:

Oral Cancer can be induced in a male Syrian hamsters painting right buccal mucosa, 3 times/ week for 16 weeks with 0.5% solution of DMBA in liquid paraffin (approximately 10 µl containing 100 µg). Tumour size, number and tumour burden of drug treated animals can be compared with that of control animals at the termination of experiment[67] .

4. MATERIALS AND METHODS

4.1. PREPARATION OF THE DRUG *ASHTA BAIRAVA CHENDOORAM:*

Drug selection:

In this dissertation, the sample of *AshtaBairavaChendooram* was taken as a compound drug for Oral Cancerand its preparation were taken from the classical Siddha literature **"*PranaRakshamirdhaSindhu*" Page no:390.**

Ingredients:

Table: 2.Mineral and Metal ingredients

S.NO	NAME OF DRUGS	CHEMICAL NAME	QUANTITY
1.	*Manosilai*	Realgar	35mg *(1 palam)*
2.	*Aridhaaram*	Orpiment	35mg *(1 palam)*
3.	*Kaantham*	MagneticOxide of Iron	35mg *(1 palam)*
4.	*Pooram*	Calomel	35mg *(1 palam)*
5.	*Lingam*	Cinnabar	35mg *(1 palam)*
6.	*Rasam*	Mercury	35mg *(1 palam)*
7.	*Kanthagam*	Sulphur	35mg *(1 palam)*
8.	*Vellaipadanum*	White Arsenic	35mg *(1 palam)*

Table: 3.Herbal ingredients

S.NO	Drug name	Botanical name	quantity
1.	*Kuppaimeni*	*Acalyphaindica*	60 ml
2	*Vetrilai*	*Piper betle*	60 ml
3	*Paruthi*	*Gossipiumherbaceum*	60 ml
4	*Vellarugu*	*Enicostemmaaxillare*	60ml
5	*Tulasi*	*Ocimum sanctum*	60ml

Collection of the Drug:

The mineral and metal ingredients of the drug were purchased in Govinda raja Mudhaliyar store, Parry's corner, Chennai-600001 and herbal ingredients were collected in and around Salem area.

Identification and Authentication of the drug:

The minerals and metals were identified and authenticated by Gunapadam experts, P.G Dept. of *Gunapadam*, GSMC, Arumbakkam, Chennai-106.

Purification process:

Purification process done as per Siddha classical literatures

1. Realgar:

Realgar(50gm)was soaked in ginger juice (*Gingiberofficinalis*- 50 ml) in the mud plate and then dried to get purified Realgar[68A].

2. Orpiment:

Orpiment (50gm) piece was buried in a heap of lime stone. Palm toddy was poured on the lime stone for 10 times and then washed and dried to get purified Orpiment[68B].

3. Magnetic Oxide of Iron:

Magnetic Oxide of Iron (50gm) was soaked in 300 ml of*Ponnavara root juice (Cassia auriculata* -300ml*)* from morning to evening for ten days. Then it was dried for another two days without adding the juice. This process was repeated two times and washed to get purified Magnetic Oxide of Iron[68C].

4. Calomel:

The poultice made of betel leaf (*Piper betle)* and pepper (*Piper nigrum)* each 8.75 gm are taken and dissolved in 1.3 litre of water. Calomel *(*50gm) is tied with a cloth and immersed in the liquid from the cross bar and heated. After the water is reduced to ¾ of its volume, the Calomel is taken out, washed with water and dried to get purified Calomel [68D].

5.Cinnabar:

Cinnabar (50 gm) was soaked in Cow's milk (50 ml) in a mud plate for one day. Next it was soaked in lemon juice (50 ml) in the same mud plate for one day. Then it was washed thoroughly with water and dried to get purified Cinnabar[68E].

6. Mercury:

Mercury - 35gram

Brick powder - 15 gm

Turmeric powder - 20 gm

Acalyphaindica juice - 1.3 litre

Mercury was triturated with finely powdered brick and then turmeric powder for one hour respectively and washed with water. Then Mercury was boiled with the juice of Acalyphaindica till the juice completely evaporates. We get purified Mercury[68F].

7. Sulphur:

Sulphur(50gm) was placed in an Iron spoon. A small quantity of cow's butter was added and the spoon was heated till the butter melts, the mixture was immersed in inclined position in cow's milk. This procedure is repeated for 30 times to get purified Sulphur. Each time fresh milk is to be used[68G].

8. White Arsenic:

White Arsenic (50gm) was powdered and triturated with lemon juice. It is made into small cakes and dried. This process is repeated for 7 times to get purified White Arsenic [68H].

Preparatory method of *AshtaBairavaChendooram:*

1. Purified Realgar - 35gm
2. Purified Orpiment - 35gm
3. Purified Magnetic Oxide of Iron - 35gm
4. Purified Calomel - 35gm
5. Purified Cinnabar - 35gm
6. Purified Mercury - 35gm

7. Purified Sulphur - 35gm
8. Purified White Arsenic - 35gm

Juice of the following herbals,

1. *Acalyphaindica* - 60 ml
2. *Piper betle* - 60 ml
3. *Gossypiumherbaceum*- - 60 ml
4. *Enicostemmaaxillare*- - 60 ml
5. *Ocimum sanctum* - 60ml

Procedure:

All the above mentioned metal and mineral ingredients were taken, powdered separately and ground well in a*kalvam* (stone mortar). *Kuppaimeni* juice was added to it ground for 3 hours. Then it was ground by adding *Vetrilai, Paruthi, Velarugu, Thulasi* respectively for 3 hours each. The mixture was made in to pellets and allowed to dry. The pellets were kept in a mud pot covered by betel leaf paste. This is covered by another pot and their mouths are sealed with seven layers of mud sealed cloth. Then it was ignited for 12 hours using *deepakkini* (small flame). Finally the clay smeared cloth was removed and the pots were separated. The *Chenduram*was found sticking to the upper pot. This was collected by a clean spoon and labeled as *AshtaBairavaChenduram (ABC).*

Storage:

The drug was stored kept in an air tight glass container.

Total chendooram - 65gm

Weight of wastage - 190gm

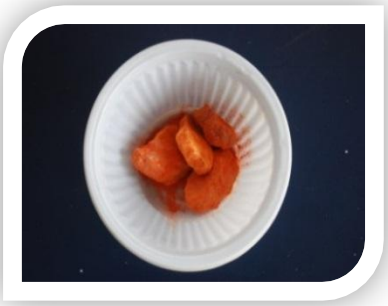
Dosage -Kundrimanialavu(130mg).

Adjuvant - honey

Indications:

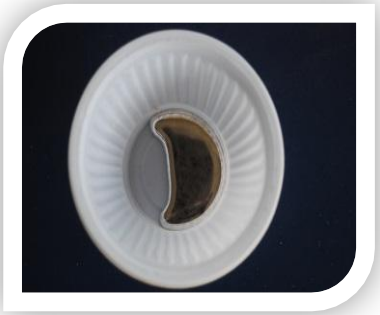
Kannaputru, Sanni 13, Kuttam, Kiranthi 9, Pilavai, Putru

METAL INGREDIENTS

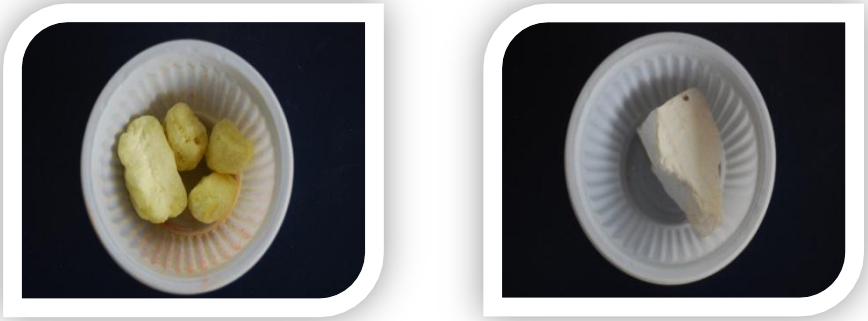


REALGAR ORPIMENT

MEGNETIC OXIDE OF IRON CALOMEL



CINNABAR MERCURY



SULPHUR WHITE ARSENIC

Figure: 1 Metal ingredient of the drug ABC

HERBAL INGREDIENT

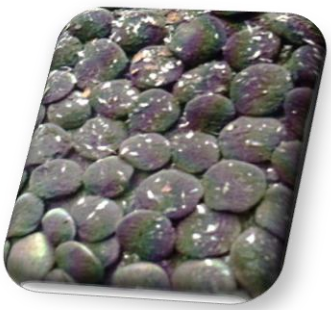
Acalyphaindica ***Piper betle***

GossipiumherbaceumEnnicostemmaaxillare

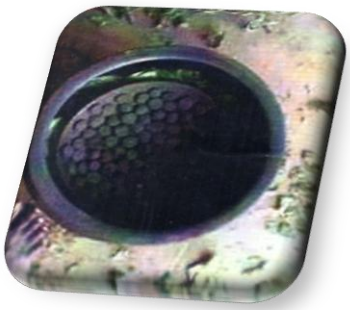
Ocimum sanctum

` **Figure:2 herbal ingredient of the drug ABC**

PREPARATORY METHOD OF DRUG



Preparation of Cakes



Arrangement in Mud Pot

Burning process

AshtaBairavaChenduram

Figure: 3 Preparatory method of drug

Analysis as per classical Ayushguideliness:

1. Floating on Water:

A pinch of *Chenduram* gently placed over water kept in a vessel, did not sink immediately. It was found that the *Chenduram*particles floated over the surface of water indicated lightness of the trial drug.

2. Lines on fingers:

*Chenduram*in well prepared form should be fine. When taken between thumb and index finger, the fine powder will fill up the lines of the finger print. A pinch of Ashta*BairavaChenduram*was taken in between the thumb and index finger and rubbed. It was found that the *AshtaBairavaChenduram*entered into the lines of the finger, and was not easily washed out from the lines, confirmed its fineness.

3. Irreversible reaction:

The well prepared *chenduram* is not reversible to its metallic state when heated with a mixture of cane jaggery, hemp powder, ghee and honey. A pinch of Ashta*BairavaChenduram*was taken and mixed with cane jaggery, ghee and honey. It was observed that the Ashta*BairavaChenduram*did not become reversible to its metallic state.

4. Tastelessness:

The well prepared *Chenduram*should be completely tasteless. Presence of any taste like sweet or bitter indicate incomplete preparation which needed another calcination process. When a small amount ofAshta*BairavaChenduram*was kept on the tip of the tongue, no specific taste was found.

5. Lusterless:

If any shining particle present in *Chenduram*, it indicates that the *chenduram*is not manufactured properly and contains incomplete form of substances like minerals, metals and other toxic substances. There should be no shining particles present in the wellmanufactured*Chenduram*. The *AshtaBairavaChenduram*was taken in a Petri bowl and observed for any lustre in daylight through magnifying glass. No lustre was observed in the *Chenduram*[69A].

4.2. STANDARDIZATION OF THE DRUG*ABC:*

Standardization of drugs helps to prove its identity and determination of its quality and potency. Standardization of the herbo mineral formulation is based on the qualitative and quantitative analysis through physico-chemical investigations and instrumental analysis. The physico-chemical analysis of the prepared herbo mineral drug have been done at Central Research Institute,Arumbakkam,Chennai and elemental analysis have been done at IIT,Chennai(FTIR,SEM,ICP-OES,XRD).

4.2.1. Physico-Chemical Investigations:

Physico-chemical investigations like pH value, loss on drying at 105°C, action on heat, Flame test and Ash test have been done at Central Research Institute, Arumbakkam, Chennai as per the guide lines of AYUSH.

pH value:

Potentiometrically pH value is determined by a glass electrode and a suitable pH meter.

Loss on Drying:

The powdered drug is dried in the oven at 100- 105°C to constant weight.

Action on heat:

A small amount of the sample is taken in a dry test tube and heated gently. If strong white fumes evolve indicate the presence of Carbonate.

Flame test:

A small amount of the sample is made into a paste with conc.HCl in a watch glass and introduced into non-luminous part of the Bunsen flame. Appearance of bluish green flame indicates the presence of Copper.

Ash Test:

A filter paper is soaked into a mixture of sample and Cobalt nitrate solution and introduced into the Bunsen flame and ignited. Appearance of yellow colour flame indicates the presence of Sodium.

4.2.2 BIO-CHEMICAL ANALYSIS

Preliminary Basic and Acidic radical studies:

Preparation of extract

10g of sample was taken in a 250 ml of clean beaker and 50 ml of distilled water was added to it. Then it was boiled well for about 10 mins. Then it was allowed to cool and filtered in a 100 ml volumetric flask and made up to 100 ml with distilled water. This preparation was used for the qualitative analysis of acidic/ basic radicals and biochemical constituents in it.

Test for Basic radicals

1. Test for Potassium

To a pinch of the *ABC* 2 ml of sodium nitrate and 2 ml of cobalt nitrate solution in 30% glacial acetic acid was added and observed for the presence of yellow precipitate.

2. Test for Calcium

To 2 ml of *ABC* extract, 2 ml of 4% ammonium oxide solution was added and observed for the formation of white precipitate**.**

3. Test for Magnesium:

To 2ml of *ABC* extract, drops of sodium hydroxide solution was added and watched for the appearance of white precipitate.

4. Test for Ammonium:

To 2ml of *ABC* extract few ml of Nessler's reagent and excess of sodium hydroxide solution are added for the appearance of brown colour.

5. Test for Sodium

Hydrochloric acid was added with a pinch of the *ABC*, made as paste and introduced into the blue flame of Bunsen burner and observed for the appearance of intense yellow colour.

6. **Test for Iron (Ferrous)**

The *ABC* extract was treated with Conc. HNO_3 and ammonium thiocyanate and waited for the appearance of blood red colour**.**

7. Test for Zinc

To 2 ml of the *ABC* extract drops of sodium hydroxide solution was added and observed for white precipitate formation.

8. Test for Aluminium

To the 2m1 of the *ABC* extract sodium hydroxide was added in drops and changes are noted.

9. Test for Lead

To 2 ml of *ABC* extract 2ml of potassium iodide solution was added and noted for yellow coloured precipitate**.**

10. Test for Copper

a. A pinch of *ABC* was made into a paste with con. Hcl in a watch glass and introduced into the non-luminous part of the flame and noted for blue colour appearance.

b. To 2 ml of *ABC* extract excess of ammonia solution was added and observed for the appearance of blue coloured precipitate**.**

11. Test for Mercury

To 2m1 of the *ABC* extract sodium hydroxide solution was added and noted for yellow precipitate formation**.**

12. Test for Arsenic

To 2 ml of the *ABC* extract 2ml of sodium hydroxide solution was added and brown or redprecipitate formation was noted.

Test for acid radicals

1. Test for Sulphate

To 2 ml of the *ABC* extract 5% of barium chloride solution was added and observed for the appearance of white precipitate.

2. Test for Chloride

The *ABC* extract was treated with silver nitrate solution and observed for the appearance of white precipitate.

3. Test for Phosphate

The ABC extract was treated with ammonium molybdate and conc. HNO_3 and observed for the appearance of yellow precipitate.

4. Test for Carbonate

The *ABC* extract was treated with conc.Hcl and observed forth appearance of effervescence.

5. Test for Fluoride & Oxalate:

To 2ml of *ABC* extract 2ml of dil.acetic acid and 2ml calcium chloride solution was added and heated and watched for cloudy appearance.

6. Test for Nitrate:

To 1 gm of the *ABC*, copper turnings was added and again conc.H_2SO_4 was added, heated and the test tube was tilted vertically down and observed for any changes[69B].

4.2.3 AVAILABILITY OF BACTERIAL LOAD:

Enumeration of bacteria by plate count – agar plating technique

The plate count technique is one of the most routinely used procedure because of the enumeration of viable cells by this method.

Principle:

This method is based on the principle that when material containing bacteria is cultured, every viable bacterium develops into a visible colony on a nutrient agar medium. The numbers of colonies therefore are the same as the number of organisms contained in the sample. [70]

Dilution:

Small measured volumes are mixed with a large volume of sterile water or saline called the diluent or dilution blank. Dilutions are usually made in multiples of ten. A single dilution is calculated as follows:

$$\text{Dilution} = \frac{\text{Volume of the sample}}{\text{Total volume of the sample and the diluent}}$$

Requirements:

- Sample or Bacterial suspension
- 9 ml dilution blanks (7)
- Sterile petri dishes (12)
- Sterile 1 ml pipettes(7)
- Nutrient agar medium (200 ml)
- Colony counter [71]

Procedure:

1. Label the dilution blanks as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}.
2. Prepare the initial dilution by adding 1 ml of the sample into a 9 ml dilution blank labelled 10^{-1} thus diluting the original sample 10 times.
3. Mix the contents by rolling the tube back and forth between hands to obtain uniform distribution of organisms.
4. From the first dilution transfer 1 ml of the suspension while in motion, to the dilution blank 10^{-2} with a sterile and fresh 1 ml pipette diluting the original specimen to 100 times.
5. From the 10^{-2} suspension, transfer 1 ml of suspension to 10^{-3} dilution blank with a fresh sterile pipette, thus diluting the original sample to 1000 times.
6. Repeat these procedures till the original sample have been diluted 10,000,000 times using every time a fresh sterile pipette.
7. From the appropriate dilutions transfer 1ml of suspension while in motion, with the respective pipettes, to sterile petri dishes. Three petri dishes are to used for each dilution.

8. Add approximately 15 ml of the nutrient medium, melted and cooled to 45^0c, to each petri dish containing the diluted sample. Mix the contents of each dish by rotating gently to distribute the cells throughout the medium.
9. Allow the plates to solidity.
10. Incubate these plates in an inverted position for 24-48 hours at 37^0c[72].

Observation: Observe all the plates for the appearance of bacterial colonies. Count the number of colonies in the plates.

Calculate the number of bacteria per ml of the original suspension as follows:

$$\text{Organisms per millimetre} = \frac{\text{Number of colonies (average of 3 replates)}}{\text{Amount of plated} \times \text{dilution}}$$

4.2.4. SOPHISTICATED INSTRUMENTAL ANALYSIS

FT-IR (Fourier Transform Infra-Red)

Model	**: Spectrum one: FT-IR Spectrometer**
Scan Range	**: MIR 450-4000 cm-1**
Resolution	**: 1.0 cm-1**
Sample required	**: 50 mg, solid or liquid.**

It is the preferred method of infrared spectroscopy. FT-IR is an important and more advanced technique. It is used to identify the functional group, to determine the quality and consistency of the sample material and can determine the amount of compounds present in the sample. It is an excellent tool for quantitative analysis[73].

Sample preparation:

In FT-IR infrared is passed from a source through a sample. This infrared is absorbed by the sample according to the chemical properties and some are transmitted. The spectrum that appears denotes the molecular absorption and transmission. It forms the molecular

fingerprint of the sample. Like the finger print there is no two unique molecular structures .the spectrum indicates the amount of material present.

FT-IR is the most advanced and the major advantage is, its

- Speed
- Sensitivity
- Mechanical simplicity
- Internally calibrated [74]

SEM (SCANNING ELECTRON MICROSCOPE)

In scanning electron microscope high-energy electron beam is focused through a probe towards the sample material. Variety of signals was produced on interaction with the surface of the sample. This results in the emission of electrons or photons and it is collected by an appropriate detector[75].

The types of signal produced by a scanning electron microscope include

- Secondary electrons
- back scattered electrons
- characteristic x-rays, light
- specimen current
- Transmitted electrons.

This gives the information about the sample and it includes external morphology, texture, its crystalline structure, chemical composition and it displays the shape of the sample [76].

ICPOES (INDUCTIVELY COUPLED PLASMA OPTIC EMISSION SPECTROMETRY)

Manufacturer: Perkin Elmer

Model: Optima 5300 DV ICP-OES Inductively Coupled Plasma Spectrometer (ICP)

Principle:

An aqueous sample is converted to aerosols via a nebulizer. The aerosols are transported to the inductively coupled plasma which is a high temperature zone (8,000–10,000ºC). The analysts are heated (excited) to different (atomic or ionic) states and produce characteristic optical emissions (lights). These releases are separated based on their respective wavelengths and their strengths are measured (spectrometry). The intensities are proportional to the concentrations of analysis in the aqueous sample. The quantification is an external

multipoint linear standardization by comparing the emission intensity of an unknown sample with that of a standard sample. Multi-element calibration standard solutions are prepared from single- and multi element primary standard solutions. With respect to other kinds of analysis where chemical speciation is relevant (such as the concentration of Ferrous Iron or Ferric Iron), only total essential concentration is analysed by ICP-OES[77].

Application:

The analysis of major and minor elements in solution samples.

Objectives:

- Determine elemental concentrations of different metals.
- Learn principles and operation of the ICP-OES instrument
- Develop and put on a method for the ICP-OES sample analysis
- Enhance the instrumental conditions for the analysis of different elements
- Probes the outer electronic structure of atoms

Mechanism:

In plasma emission spectroscopy (OES), a sample solution is presented into the core of inductively coupled argon plasma (ICP), which generates temperature of approximately 8000°C. At this temperature all elements become thermally excited and emit light at their characteristic wavelengths. This light is collected by the spectrometer and passes through a diffraction grating that serves to resolve the light into a spectrum of its essential wavelengths. Within the spectrometer, this deflected light is then collected by wavelength and amplified to yield an strength of measurement that can be converted to an elemental concentration by comparison with standardization values.

The Inductively coupled plasma optical emission spectrometric (ICP-OES) analysis was done in SAIF, IIT MADRAS,Chennai-36 using Perkin Elmer Optima 5300 DV[78].

Sample preparation:

Inductively Coupled Plasma Spectroscopy techniques are the so-called "wet" sampling methods whereby samples are introduced in liquid form for analysis.

100 mg *ABC* was occupied in a clean, dry test tube. To this, 3 ml Nitric acid was added and mixed well and allowed for few minutes until the reactions were completed. And then, 25 ml of Refined water, was added to prepare digested solution. The digested sample solution was shifted into plastic containers and labeled properly. It was completed in Bio-chemistry lab, Govt. Siddha Medical College, Chennai-106.

XRD (X-RAY POWER DIFFRACTION)

The XRD uses x-ray radiation.

Procedure:

Sample Preparation:

XRD can be done on a number of different kind of samples.The sample is a crystalline powder that had pressed into the sample holder, and it has hold at an angle of 45 degrees. Solid small volumes of sample taped on the microscope slide glass or thin films deposited on a substrate but will have varying degree of effectiveness. The result of the sample is based on the sample's crystalline nature .

Check the alarm light on the right hand side of the instrument and fill the XRD log spreadsheet on desktop. [79]

FTIR (Fourier Transform Infrared Spectroscopy)

Figure: 4 - FTIR INSTRUMENT

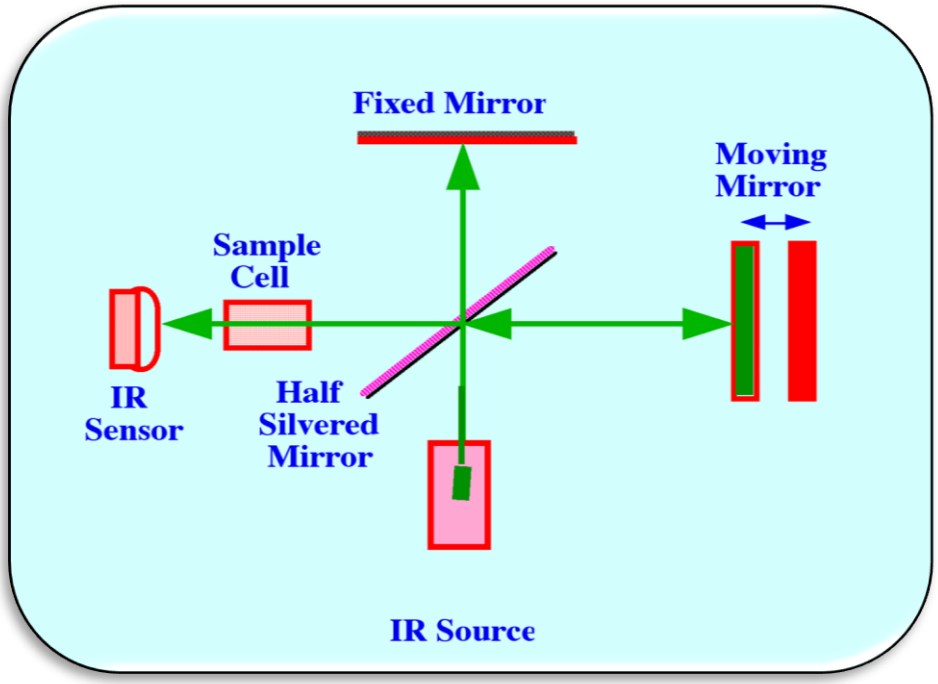


Figure:5 - FTIR MECHANISM

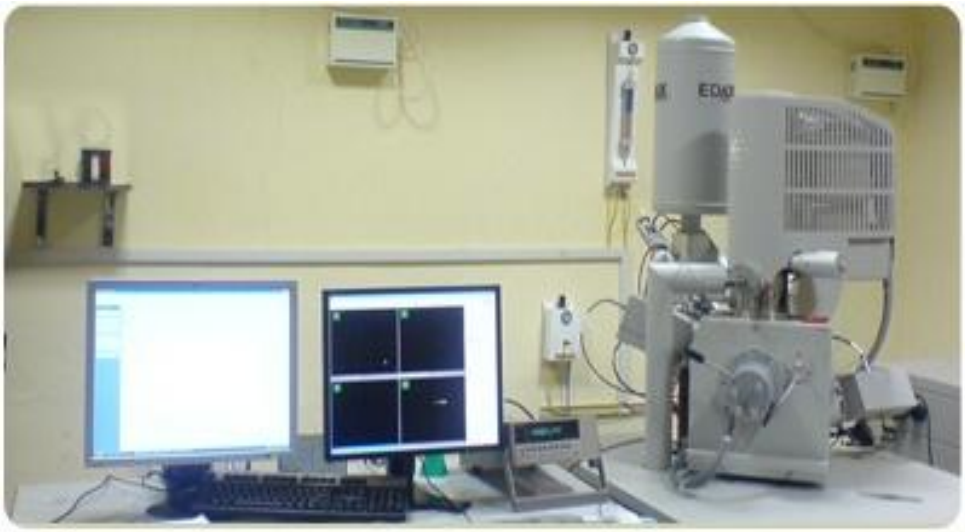


Figure: 6 - SEM- SCANNING ELECTRON MICROSCOPE

SEM INSTRUMENT

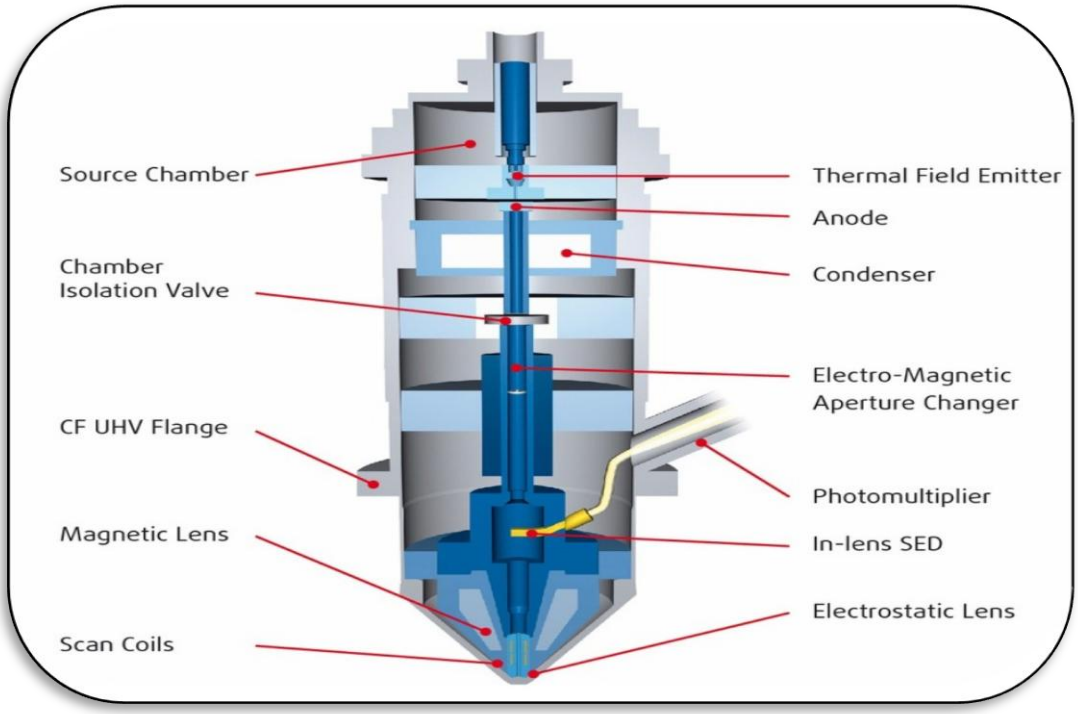


Figure: 7-SEM MECHANISM

Figure: 8-ICP-OES ANALYSER (Perkin Elmer Optima 5300 DV)

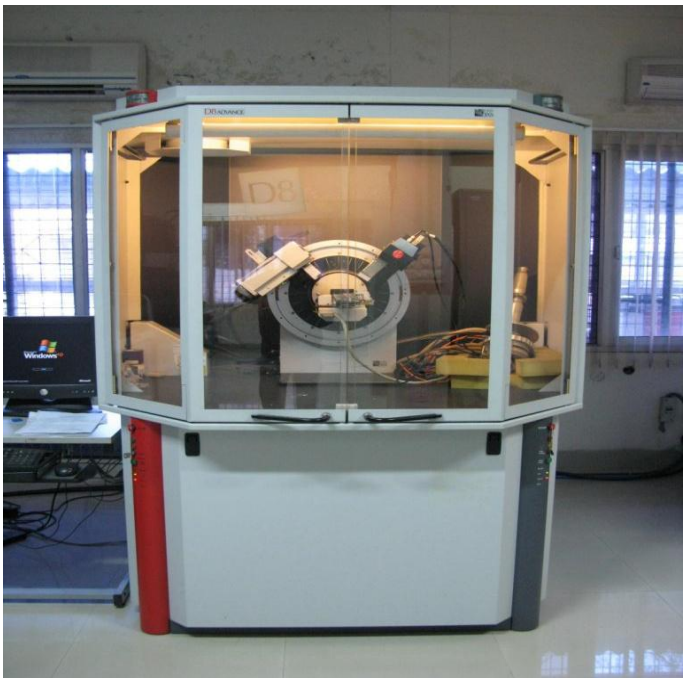


Figure: 9-X-Ray Diffraction analysis(XRD)

4.3. TOXICOLOGICAL STUDIES

INTRODUCTION:

The acute toxic class method is a stepwise procedure with the use of 3 animals of a single sex per step. Depending on the mortality and / or the moribund status of the animals, on average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance. Morbid animals or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on test. The method allows for the determination of an LD50 value only when at least two doses result in mortality higher than 0% and lower than 100%.

PRINCIPLE:

It is the principle of the test that based on a stepwise procedure with the use of a minimum number of animals per step, sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex. Absence or presence of compound-related mortality of the animals dosed at one step will determine the

next step, i.e.; − no further testing is needed − dosing of three additional animals with the same dose − dosing of three additional animals at the next higher or the next lower dose level. The method will enable a judgment with respect to classifying the test substance to one of a series of toxicity classes.

4.3.1.ACUTE ORAL TOXICITY – OECD GUIDELINES - 423

Acute toxicity study was carried out as per OECD guideline (Organization for Economic Co - operation and Development, Guideline-423.

CPCSEA [Approval no:IAEC/XLIV/29/CLBMCP/2014]

Animal: Healthy Wistar albino female rat weighing 200–220 gm

Studies carried out on three female rats under fasting condition, signs of toxicity were observed for every one hour for first 24 hours and every day for about 14 days from the beginning of the study [80].

METHODOLOGY

Selection of animal species:

The preferred rodent species is rat, although other rodent species may be used. Healthy young adult animals of commonly used laboratory strain Swiss albino rat was obtained from Animal house of King's institute, Guindy, Chennai. Females should be nulliparous and non-pregnant. Each animal at the commencement of its dosing should be between 8 and 12 weeks old and its weight should fall in an interval within±20 % of the mean weight of the animals. The studies were conducted in the animal house of C.L.BaidMetha college of pharmacy, Duraipakkam, Chennai.

Housing and feeding conditions:

The temperature in the experimental animal room should be 22ºC (+3ºC). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hrs light, 12 hrs dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be grouped and tagged by dose, but the number of animals per cage must not interfere with clear observations of each animal[81] .

Preparation of animals:

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions[82].

EXPERIMENT PROCEDURE:

Administration of dose

Ashta*BairavaChendooram*prepared as per the classical Siddha literature was suspended in 2% CMC with uniform mixing and was administered to the groups of Wistar albino rats. It is given in a single oral dose by gavage using a feeding needle. Animals were fasted prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered. After the substance has been administered, food was withheld for further 3-4 hours. The principle of laboratory animal care was followed. Observations were made and recorded systematically and continuously observed as per the guideline after substance administration.

The visual observations included skin changes, mobility, aggressively, sensitivity to sound and pain, as well as respiratory movements. They were deprived of food, but not water 16–18 h prior to the administration of the test suspension. Finally, the number of survivors was noted after 24 h and these animals were then maintained for a further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality.

Number of animals and dose levels

Since this test drug has been under practice for long time and likely to be non-toxic, a limit test at one dose level of 2000 mg/kg body weight will be carried out with 6 animals (3 animals per step).

Duration of Study : 48 hrs

Evaluation : 14 Days

Limit test

The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity only above

regulatory limit doses. A limit test at one dose level of 2000 mg/kg body weight was carried out with three animals per step. The test substance-related mortality was not produced in animals, so further testing at the next lower level need not be carried out.

Observations

- The animals were observed individually after dosing at least once during the first 30mins and periodically during the first 24 hrs.
- Special attention: First 1-4 hrs after administration of drug.
- It is observed daily thereafter for a total of 14 days, except when they needed to be removed from the study and killed humanely for animal welfare reasons or are found dead.

a. Mortality

Animals will be observed intensively at 0.5, 2.0, 4.0, 6.0, 12.0, 24.0 and 48.0 hour following drug administration on day 1 of the experiment and daily twice thereafter for 14 days.

b. Body weight

Body weights will be recorded at day: -1, day 1, 2, 7 and 14 of the study

c. Cage-side observation

These include changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

d. Gross necropsy

All animals (including those which die during the test period are removed from the study) will be subjected to gross necropsy. Gross necropsy includes examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities and their contents, brain, eye, thymus, lungs, heart, spleen, liver, kidneys, adrenals, testes and uterus of all animals

Histopathology

Microscopic examination will be carried out in organs to show the evidence of any toxicity in gross pathology.

Data and reporting

All the data were summarised in tabular form showing the animals used, number of animals displaying signs of toxicity, the number animals found dead during the test or killed

for humane reasons, a description and the time course of toxic effects and reversibility, and necroscopic findings.

Test substance and Vehicle

In order to ensure the uniformity in drug distribution in the medium the suspension was made by mixing Ashta*BairavaChendooramwith* 2% CMC solution and it was found suitable for dose accuracy.

Justification for choice of vehicle

The vehicle selected as per the standard guideline is pharmacologically inert and easy to employ for new drug development and evaluation technique.
(Schlede E., Mischke U., Diener W. and Kayser D 1992;66: 455-470)

4.3.2.REPEATED DOSE 28 DAYS ORAL TOXICITY STUDY OF AshtaBairavaChenduram in RATS – (OECD-407 guidelines)

Justification for Dose Selection

The results of acute toxicity studies in Wistar albino rats indicated thatAshta*BairavaChendooram* was non-toxic and no behavioural changes was observed up to the dose level of 2000 mg/kg body weight. On the basis of body surface area ratio between rat and human, the doses selected for the study were100mg/kg, 200 mg/kg and 400 mg/kg body weight. The oral route was selected for use because oral route is considered to be a proposed therapeutic route.

Preparation and administration of dose

Ashta*BairavaChendooram*at three doses respectively was suspended in 2 ml of 2% CMC in distilled water. It was administered to animals at the dose levels of 100, 200 and 400 mg/kg. The test substance suspensions were freshly prepared every day for 28 days. The control animals were administered vehicle only. Administration was by oral (gavage), once daily for 28 consecutive days.

METHODOLOGY

Randomization, Numbering and Grouping of Animals

Ten rats (Five Male and Five Female) were in each group randomly divided into four groups for dosing up to 28 days. Animals were allowed acclimatization period of 7 days to laboratory conditions prior to the initiation of treatment. Each animal was fur marked with picric acid. The females were nulliparous and non-pregnant.

OBSERVATIONS

Experimental animals were kept under observation throughout the course of study for the following:

Body Weight*:* Weight of each rat was recorded on day 0, at weekly intervals throughout the course of study and at termination to calculate relative organ weights. From the data, group mean body weights and percent body weight gain were calculated.

Clinical signs: All animals were observed daily for clinical signs. The time of onset, intensity and duration of these symptoms, if any, were recorded.

Mortality*:* All animals were observed twice daily for mortality during entire course of study.

Functional Observations: At the end of the 4^{th} week exposure, 'sensory reactivity' to graded stimuli of different types (auditory, visual and proprioceptive stimuli), 'motor reactivity' and 'grip strength' were assessed.

Laboratory Investigations: Following laboratory investigations were carried out on day 29 in animals fasted over-night. Blood samples were collected from orbital sinus using Sodium heparin (200IU/ml) for Blood chemistry and Potassium EDTA (1.5 mg/ml) for Haematology as anticoagulant. Blood samples were centrifuged at 3000 r.p.m. for 10 minutes. On 28th day of the experiment, 24 h urine samples were collected by placing the animals in the metabolic cage with free access to tap water but no feed was given.

The urine was free from fecal contamination. Toluene is used as a preservative while collecting the sample. The sediments present in the urine were removed by centrifugation and the collected urine was used for biochemical estimations. On 29th day, the animals were fasted for approximately 18 h, then slightly anesthetized with ether and blood samples were collected from the retro-orbital plexus into two tubes: one with EDTA for immediate analysis of haematological parameters, the other without any anticoagulant and was centrifuged at 4000 rpm at 4 °C for 10 minutes to obtain the serum. Serum was stored at 20 °C until analyzed for biochemical parameters.

Haematological Investigations: Blood samples of control and experimental rats was analyzed for hemoglobin content, total red blood corpuscles (RBC), white blood corpuscles (WBC) count and packed cell volume (PCV).

Biochemical Investigations: Serum was used for the estimation of biochemical parameters. Samples of control and experimental rats were analyzed for protein, bilirubin, urea, BUN, creatinine, triglyceride, cholesterol and glucose levels was carried using standard methods. Activities of Glutamate oxaloacetate transaminase/ Aspartate aminotransferase (GOT/AST), Glutamate pyruvate transaminase/ Alanine amino transferase (GPT/ALT) and Alkaline phosphatase were estimated as per the colorimetric procedure.

Urine analysis: Urine samples were collected on end of treatment for estimation of normal parameters. The estimations were performed using appropriate methodology.

Necropsy*:*All the animals were sacrificed on day 29. Necropsy of all animals was carried out and the weights of the organs including liver, kidneys, spleen, brain, heart, and lungs were recorded. The relative organ weight of each animal was then calculated as follows;

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of animal on sacrifice day (g)}} \times 100$$

Histopathology: Histopathological investigation of the vital organs was done. The organ pieces (3-5µm thick) of the highest dose level of 400 mg/kg were preserved and were fixed in 10% formalin for 24 h and washed in running water for 24 h. Samples were dehydrated in an auto technicon and then cleared in Benzene to remove absolute alcohol. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a cubical block of paraffin made by the "L" moulds. It was followed by microtome and the slides were stained with Haematoxylin-Eosin. The organs included heart, kidneys, liver, ovary, pancreas, brain, spleen and stomach, of the animals were preserved they were subjected to histopathological examination.

Statistical analysis: Findings such as clinical signs of intoxication, body weight changes, food consumption, hematology and blood chemistry were subjected to One-way ANOVA followed by Dunnet'smulticomparision test using a computer software programme GRAPH PAD INSTAT-3 version.

4.4PHARMACOLOGICAL STUDY:

4.4.1 INVITRO ANTI- ORALCANCER ACTIVITY DETERMINATION BY MTT ASSAY

Materials and methods:

KB(skin cancer cells) was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbeccos modified Eagles medium (Gibco, Invitrogen).

The KB cell lines was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, Sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate:

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 210% growth medium, 100µl cell suspension ($5x10^4$ cells/well) wasseeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of plant extracts and compound stock:

1 mg of compound sample was added to 1ml of DMEM and dissolved completely by cyclomixer. After that the extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

Cytotoxicity Evaluation: After 24 hours the growth medium was removed, freshly prepared each plant extracts in 5% DMEM(Dulbecco's modified eagle medium) were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 100µl of 5% MEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator.

Cytotoxicity Assay by Direct Microscopic observation:

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method:

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization..

After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 200µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize theformazan crystals). The absorbance values were measured by using microplate reader at a wavelength of 540 nm[83].

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples x 100}}{\text{Mean OD of control group}}$$

4.4.2. In vitro Anti- tumour activity

Cell culture materials

OSCC cell lines were purchased from American Tissue Collection Centre (ATCC) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (DMEM/F12) (Sigma-Aldrich, USA).HOS cell lines were purchased from ATCC and were maintained in DMEM high glucose. Culture media was supplemented with 10% Foetal Bovine Serum (FBS) and 1% penicillin/streptomycin.

Cell apoptosis assay by flow cytometry

Cellular apoptosis was determined using the AnnexinV-FITC Apoptosis Detection Kit I (Clontech Laboratories Inc, USA) according to the manufacturer'sprotocol. OSCC and HOS cell lines were cultured at 6 × 10cells/ml and seeded in 60 mm dish. The cells were treated with free medium containing various concentrations of ABC for 6, 12 and 24 hour. Cells were harvested by trypsinization, then washed twice with cold PBS and centrifuged at 1000 rpm. About 1 × 10^5 -1×10^6cells were then re suspended in 400 µl1×binding buffer, centrifuged again at 1000 rpm for 5 minutes and then supernatant was removed. Cells were re-suspended in 200 µl 1× binding buffer and transferred to a sterile flow cytometry glass tube. Five µl Annexin V-FITC and 10 µl propidium iodide were added and then incubated in the dark at room temperature. Cells were analyzed by flow cytometer at 488 nm. The distribution of cells was analyzed using Cell Quest software (Becton-Dickinson) in the flow

cytometer within 1 hour of staining. Data from 10,000 cells was collected for each data file. Apoptotic cells were identified as Annexin V-FITC-positive and P-negative cells.

4.4.3.ANTIOXIDANT ACTIVITY:

Method:

After the end of sub-acute toxicity study, the intermediate dosage group of animals were sacrificed and organs such as liver and kidneys were excised out and analyzed for oxidative stress markers. The concentration of oxidative stress markers such as Lipid peroxide, Glutathione, Glutathione peroxidase and Catalase were analyzed. Lipid peroxides (Thiobarbituric Acid Reactive Substances – TBARS) in tissues were assayed by the method of Yagi. [84] The colour formation with Thiobarbituric acid (TBA) was used as index. Reduced glutathione (GSH) was estimated by the method of Ellman in which yellow colour developed when Dithionitro-bis-benzoic acid (DTNB) added to the compounds sulfhydryl groups. [85]Glutothione peroxidase (GPx) estimated by the method of Rotruck et al, 1973 in which H_2O_2 reduced to water whereas organic hydroperoxides reduced to alcohol at the expense of GSH. [86][87] The activity of Catalase (CAT) was determined by the method of Sinha. [88] In this assay, Dichromate in Acetic acid heated in the presence of Hydrogeperoxide converted to Perchromic acid and then to Chromic acetate. The formed chromic acetate was measured at 620 nm.

FREE RADICAL SCAVENGING ACTIVITY:

DPPH ASSAY (2, 2-diphenyl -1-picrylhydrazyl)

The radical scavenging activity of extracts was determined by using DPPH assay according to Chang et al[2001].The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference[89].

PRINCIPLE

1,1-Diphenyl-2-picryl hydrazyl is a stable free radical with red colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as, DPPH + [H-A] →DPPH-H + (A)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

REAGENT PREPARATION

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol.

PROCEDURE

Different volumes (2.5µl - 40µl) of plant extracts were made up to a final volume of 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

CALCULATION

$$\% \text{ inhibition} = \frac{\text{Control-test}}{\text{control}} \times 100$$

5. RESULTS AND DISCUSSION

Many studies have been carried out to bring the efficacy and potency of the drug *AshtaBairavaChenduram.* The study includes literary collections, physicochemical and phytochemical analysis, instrumental analysis, toxicological study and pharmacological study. The drug *AshtaBairavaChenduram* has been selected for Anti-cancer activity in reference with the text **"*PranaRakshamardhaSindhu*"**Literary collections about the drug from various text books were done. Siddha literatures related to the drug bring the evidence and importance of its utility in treating the cancer.

- Botanical aspect explains the identification, description, active principle and medicinal uses of the plants.
- Gunapadam review brings the effectiveness of the drug in treating cancer.
- Pharmaceutical review describes about the *chenduram* and its properties.
- The pharmacological review explains about the methodology of Anti-cancer Activity and the drugs used.

Modern and siddha aspect of the disease was also reviewed.

Results of Siddha Standardization –Interpretation

Colour:

It is blackish brown in colour. The absence of shining indicates there is no free form of metals.

Floating on water:

*AshtaBairavachenduram*floats on water. It is due to its less specific gravity. So, it possess the property of*chenduram*.

Finger print test:

AshtaBairavachenduram impinged on the crevices of finger. This indicates the particles are fine and it is in micro size.

Lustreless& tasteless:

It is lustreless and tasteless

Table: 4.Siddha Standardization result of *ABC*

S.No	Parameter	Result of ideal *chenduram*	Result of *ABC*	Interpretation
1	Colour	Blackich	Blackish brown	*Chenduram*
2	Floating on water	Floats on water	floats on water	Lightness of drug
3	Finger print test	Impinged in the furrow of fingers	Impinged in the furrow of fingers	
4	Lustre	Lustreless	Lustreless	Change of specific metallic character of raw material after ingnation.
5	Taste	No specific taste	No specific taste	Change of specific metallic character of raw material after ingnation.

Table: 5.Physical characterization of *AshtaBairavaChenduram*

Parameter	Result
Colour	Brown in colour
State of the drug	Powder
Consistency	Fine powder
Solubility	Sparingly soluble in water, DMSO. Well soluble in acids (Hcl and H_2So_4)
Sense on touch	Fine
Sense on taste	Tasteless
Sense of smell	No significant smell is observed

Table:6.PHYSICOCHEMICAL ANALYSIS

S.NO	Parameter	Result
1.	Specific gravity	0.976
2.	Ph	5.57%
3.	Flame test	+
4.	Ash test	-
5.	Particle size	Completely passes through sieve no.120
6.	Loss on drying at 105 degree Celsius	3.04%
7.	Total ash	26.87%
8.	Water soluble ash	4.27%
9.	Acid insoluble ash	0.82%

Interpretation

The physicochemical analysis of the drug result reveals the Loss on drying, pH, Total Ash, water soluble ash and Acid insoluble ash.

Discussion on physic- chemical parameters

Solubility

- Solubility is one of the important parameters to attain desired concentration of drug in systemic circulation the required pharmacological response.
- The oral bioavailability depends on several factors including aqueous solubility, drug permeability etc.
- The most frequent causes of low oral bioavailability are attributed to poor solubility and low permeability
- **ABC** is soluble in major solvents, sparingly soluble in some of the solvents thereby it proves its efficiency of solubility in the stomach indirectly, increased in bio-availability.

pH (potential hydrogen)

- *AshtaBairavaChenduram* shows slightly acidic pH.
- This pH level plays a role in enzyme activity by maintaining the internal environment thus regulating the homeostasis.
- It is also an important factor for drug absorption. Because of the acidic nature, the drug is more readily absorbed in an acid medium like stomach which enhance the bioavailability of the drug.

Specific gravity

- The trial drug *AshtaBairavaChenduram* shows specific gravity which is lesser than water. It shows its nature of absorption.

Flame test

- Water blue colour flame was found which indicates presence of amount of arsenic.

loss on drying

- The low moisture content of ABC indicates that it has long shelf life.

- ❖ Moisture increase can adversely affect the active ingredient; But *AshtaBairavaChenduram* moisture doesn't damage it.
- ❖ So the low moisture content of ABC offers maximum microbial stability.

Ash Values

- ❖ Total Ash value- low total ash value ABC richness of organic substances.
- ❖ These organic compounds are responsible for the mineral supplements and therapeutic effect of *AshtaBairavaChenduram.*

Acid insoluble ash

- ❖ Lower the acid insoluble value better will be the drug quality.
- ❖ The drug possesses a low value (0.82%) of acid insoluble ash indicating that the preparation did not contain any sand, dust and stones.

Water soluble ash

- ❖ Decreased water soluble ash value (4.27%) indicates easy facilitation of diffusion and osmosis mechanisms.
- ❖ The wavenumbers from 4000 cm-1 to 1500 cm-1 gives details for identification of functional group.
- ❖ The wavenumber from 1500 cm-1 to 400 cm-1 provides particulars about molecular fingerprint.
- ❖ The above result showed the presence of functional group like alcohols, alkanes, amides in*AshtaBairavaChenduram*.
- ❖ They may be responsible for the presence of anticancer action of ABC in Oral cancer. [90]

Table: 7.Results of basic radicals studies

S.NO	Parameter	Observation	Result
1	Test for Potassium	-	Absent
2	Test for Calcium	Formation of white colour precipitate	Present
3	Test For Magnesium.	-	Absent

4	Test For Ammonium	-	Absent
5	Test For Sodium		Absent
S.NO	**Parameter**	**Observation**	**Result**
6	Test for Iron (Ferrous)		Absent
7	Test For Zinc	-	Absent
8	Test For Aluminium		Absent
9	Test For Lead	-	Absent
10	Test for Copper	-	Absent
11	Test For Mercury	Formation of yellow precipitate	Present
12	Test for Arsenic	Formation of brownish red precipitate	Present

Interpretation

Calcium:

More recent studies were conflicting, and one that was positive for effect (Lappe, et al.) did control for a possible anti-carcinogenic effect of vitamin D, which was found to be an independent positive influence from calcium-alone on cancer risk.A randomized controlled trial found that 1400–1500 mg supplemental calcium and 1100 IU vitamin D3 reduced aggregated cancers with a relative risk. [91]

Ca^{2+} play a dual role in this process considering its involvement in proliferation/activation and apoptosis of both cancer and immune cells. Ca^{2+} for a productive efficient immune response against cancer. [92]

Mercury:

Miles (1926) introduced perchloride of mercury as an antiseptic agent in rectal surgery. Goligher (1951), Morgan (1955) and Keynes (1961) introduced the technique of flushing the colon and rectum in restorative cancer surgery[93].

Royle (1964) described alleged mercury intoxication after using 200 ml of 1: 500 perchloride of mercury solution as an anti-cancer agent in renal surgery.In all cases the uptake of mercury into the blood has been well below the toxic levels defined by Lane (1954)[94].

Studies of the urinary output have confirmed the safety of this technique. It is therefore concluded that mercury perchloride is a safe anti-cancer agent when used as described in large bowel surgery[95].

Arsenic:

During the 18th and 19th centuries, a number of arsenic compounds were used as medicines. In that Arsenic trioxide has been used in a variety of method of treatment over the past 500 years, but most commonly used in the treatment of cancer.

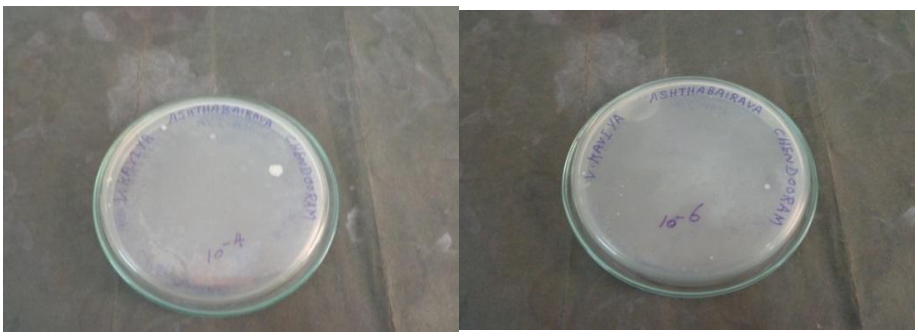
In 2000, the FDA approved this compound for the treatment of acute promyelocyticleukemia that is resistant to ATRA. Recently new research has been completed in locating tumours using arsenic-74 (a positron emitter). The advantages of using the arsenic -74 isotope instead of the formerly used iodine-124 isotope is that the signal in the PET scan is clearer as the body tends to carry iodine to the thyroid gland producing a lot of noise [96].

Table: 8.Result of acid radical studies

S.NO	Parameter	Observation	Result
1.	Test for Sulphate		Present
2.	Test for Chloride	Formation of white precipitate	Present
3	Test for Phosphate	-	Absent
4	Test for Carbonate	-	Absent
5	Test for fluoride &oxalate	-	Absent
6	Test For Nitrate	-	Absent

Anti-Microbial load Result

Bacterial dilutions



Fungal dilutions

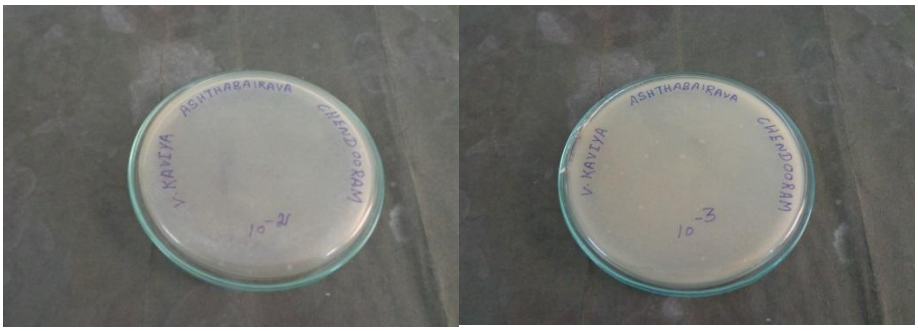


Figure:10- Bacterial and Fungal Dilutions

These herbo-metal drug are prepare from plant material they are prone to contamination. The contamination of herbal drugs by micro-organism not only cause bio deterioration but also reduces the efficacy of drugs.

The toxin produces by microbes makes herbal drugs unfit for human consumption because the contaminated drug may develop unwanted disease instead of disease being cured.

Here, the contaminations of chenduram have been examined by bacterial and fungal load.

- Total bacterial load in 10^{-4} dilution is 8 and in 10^{-6} dilution is 2.
- Total fungal load in 10^{-2} dilution is 5 and in 10^{-3} dilution is 2.

Here, the contamination of ABC is within the WHO norms. Hence, the drug is collected, prepared, stored and packed and decontaminated prior to formulation.

Result of Anti-Microbial load:

Table: 9

Bacteria		**Fungi**	
10^{-4}	**10^{-6}**	**10^{-2}**	**10^{-3}**
8	**2**	**5**	**2**

FT-IR (Fourier Transform Infra-Red)

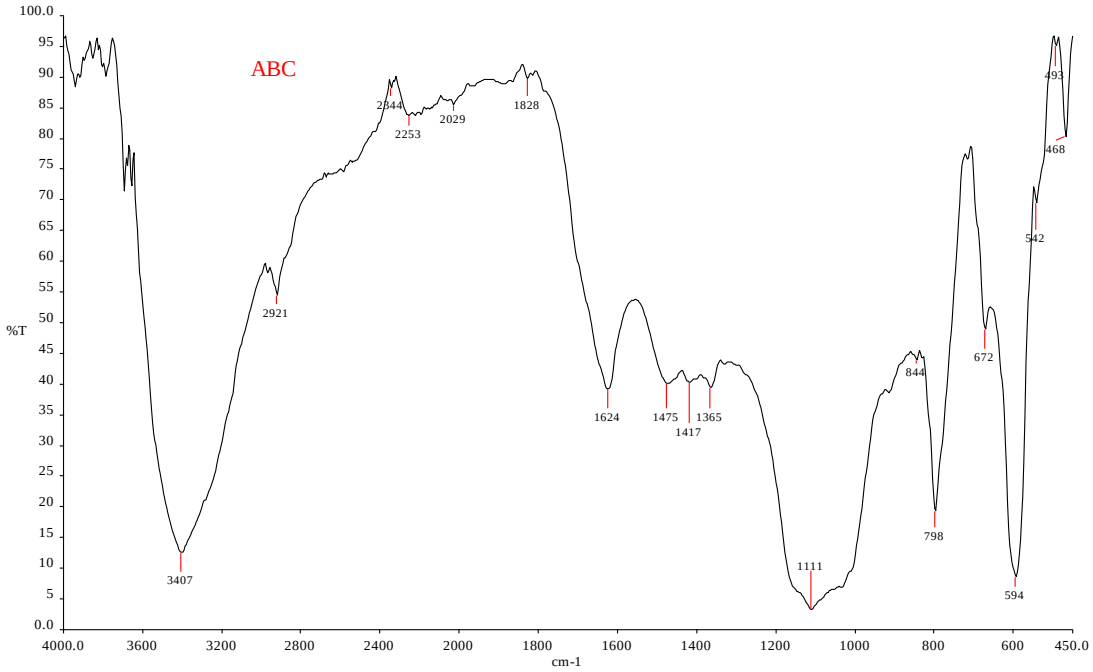


Figure: 11. Graph image of FT-IR analysis

Table: 10. Characteristic IR Absorption Frequencies of Organic Functional Groups of ABC

Characteristic IR Absorption Frequencies of Organic Functional Groups			
Functional Group	**Type of Vibration**	**Characteristic Absorptions (cm-1)**	**Intensity**
Alcohol			
O-H	(stretch, H-bonded)	3407	strong, broad
Alkane			
-C-H	Bending	1365	Variable
Alkene			
=C-H	Bending	798	Medium
=C-H	Bending	844	Strong
C=C	Stretch	1624	Variable
Alkyl Halide			

Functional Group	Type of Vibration	Characteristic Absorptions (cm-1)	Intensity
C-Br	Stretch	594	Strong
C-Br	Stretch	542	Strong
Aromatic			
C-H	Stretch	1417	medium-weak, multiple bands
C-H	Stretch	1475	medium-weak, multiple bands
Carbonyl			
C=O	Stretch	1828	Strong
Nitrile			
CN	Stretch	2253	Medium
Carboxyl			
OH-C-O	Stretch	2344	strong, two bands

DISSCUSSION:

- The wavenumbers from 4000 cm-1 to 1500 cm-1 gives details for identification of functional group.
- The wavenumber from 1500 cm-1 to 400 cm-1 provides particulars about molecular fingerprint.
- The above result showed the presence of functional group like alcohols, alkanes, amides in *AshtaBairavaChenduram.*
- They may be responsible for the presence of anticancer action of ABC in Oral Cancer

OH

- OH group of *AshtaBairavaChenduram* has higher potential towards inhibitory activity against microorganisms[97].

Phenols

- Phenols of ABC possess highly Anti-Oxidant property which enhances its effect against the disease
- The effect of phenols is currently of great awareness due to their anti-oxidative and possible anti-carcinogenic activities.
- Free radicals react easily with phenols to abstract the hydrogen atom from the OH group. Phenolic acids and flavonoids also work as reducing agents, free radical scavengers and quenchers of single oxygen formation[98].
- Phenolic acids components take part important roles in the control of cancer and other human diseases.
- Phenols are the most important groups of secondary metabolites and bioactive compounds. Hydroquinone is one of the phenolic group inhibits the free radical reactions. (cho7 Alchohol HTI) They are also an antioxidant substance capable of scavenging free superoxide radicals, anti-aging and reducing the risk of cancer.
- Phenolic and flavonoids possess diverse biological activities, for example, antiulcer, anti-inflammatory, antioxidant, cytotoxic and antitumor, antispasmodic and antidepressant activities [99].

Alkanes

- Alkane derivative like bis (4-amino-5-mercapto-1,2,4-triazol-3-yl) possess anti- cancer activity[100].

Carboxylic acid

- Benzene-poly-carboxylic Acid Complex (BP-CI) is a novel anticancer complex against human cancer cells.
- Docosahexaenoic acid (DHA) is an omega-3 fatty acid. Its structure is a carboxylic acid (-oic acid) with a 22- carbon chain (Docosa-is Greek for 22) and six (hexa-) cis double bounds[101].
- DHA was revealed to increase the efficacy of chemotherapy in prostate cancer cells and a chemo protective effect in a mouse model was reported[102].
- It may also be used as a non- toxic adjuvant to increase the efficacy of chemotherapy.
- In mice, DHA was found to reduce growth of human colon carcinoma cells

- The cytotoxic effect of DHA was caused by decrease in cell growth regulators[103].

Ether:

- Certain ether lipids such as 1-0-octadecyl-2-0 methyl-rec-glycero-3-phosphocholine represent a new class of antineoplastic agents. These ether lipids have been shown to be cytotoxic for a wide variety of tumors[104].

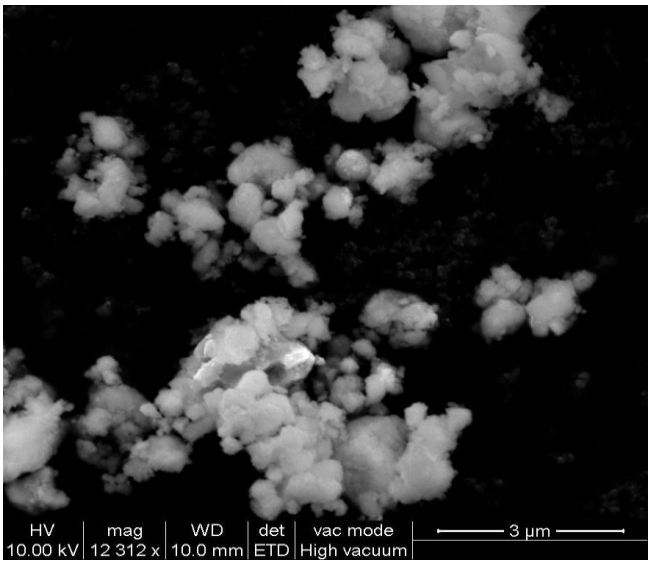


Figure: 12(a) Image of the drug by SEM analysis

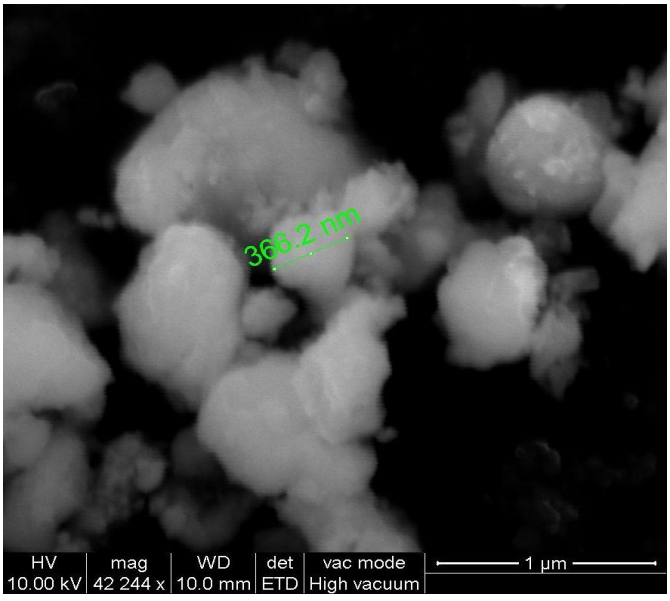


Figure: 12(b) Image of the drug by SEM analysis

Principle:

Nanoparticles, according to the American Society for Testing and Materials (ASTM) standard definition, are particles with lengths that range from 1 to 100 nm in two or three dimensions.

Advantages of nanoparticles:

- Enhancing solubility of hydrophobic drugs,
- Prolonging circulation time,
- Minimizing nonspecific uptake,
- Preventing undesirable side effects,
- Improving intracellular penetration,
- Specific cancer targeting [105]

The test drug ABC contains nanoparticles.

- The presence of nanoparticles in the drug results in a better bioavailability and facilitates absorption.
- Nanotechnology a promising way from cancer management towards cancer elimination.

Results and Interpretation of SEM analysis:

The morphology of the ABC drug can be determined by SEM (FEI Quanta). A representative portion of each sample must be sprinkled onto a double side carbon tape and mounted on aluminium stubs, in order to get a higher quality secondary electron image for SEM examination. We have observed from SEM photographs that particles are spherical in shapes and sizes are in the range from 1 micron to 3 microns. Although the particle sizes of different batches showed similarity, it seems that these particles are aggregates of much smaller particles. When dispersed in an aqueous medium, these preparations form a negatively charged hydrophobic particle suspension. This hydrophobicity gives these particles a tendency to aggregate together to form larger particles. This ABC exhibited larger sizes and agglomeration of the particles. Therefore, the comparatively larger size may be due to the agglomeration of the particles by repeated cycles of calcinations involved in preparation [106].

Table: 11. ICP-OES RESULTS OF *ASHTA BAIRAVA CHENDURAM*

S. no	Elements	Detected levels
1.	Phosphours	18.541 mg/L
2.	Calcium	14.150 mg/L
3.	Sulphur	82.514 mg/L
4.	Iron	1.330 mg/L
5.	Mercury	0.982 mg/L
6.	Potassium	20.821 mg/L
7.	Sodium	103.110 mg/L
8.	Nickel	BDL
9.	Lead	BDL
10.	Arsenic	BDL
11	Cadmium	BDL

DISSCUSSION

Table: 12.AYUSH guidelines limit for heavy metals is as follows;

HEAVY METAL LIMIT	MAXIMUM PERMISSIBLE
Arsenic (As)	3 ppm (3 mg/kg)
Cadmium (Cd)	0.3 ppm (0.3 mg/kg)
Lead (Pb)	10 ppm (10 mg/kg)
Mercury (Hg)	1 ppm (1 mg/kg)

The Inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES) results showed that the heavy metals lead, Arsenic and cadmium were found below detection level. Mercury (0.321 ppm) was found within the permissible level in *AshtaBairavaChenduram.* Hence it may be safe for human consumption. It also shows the presences of physiologically important mineral like Calcium, Iron, Sodium, Phosphate, Magnesium, Potassium, Sulphur and Phosphorous [107].

XRD –Result of *AshtaBairavaChenduram*

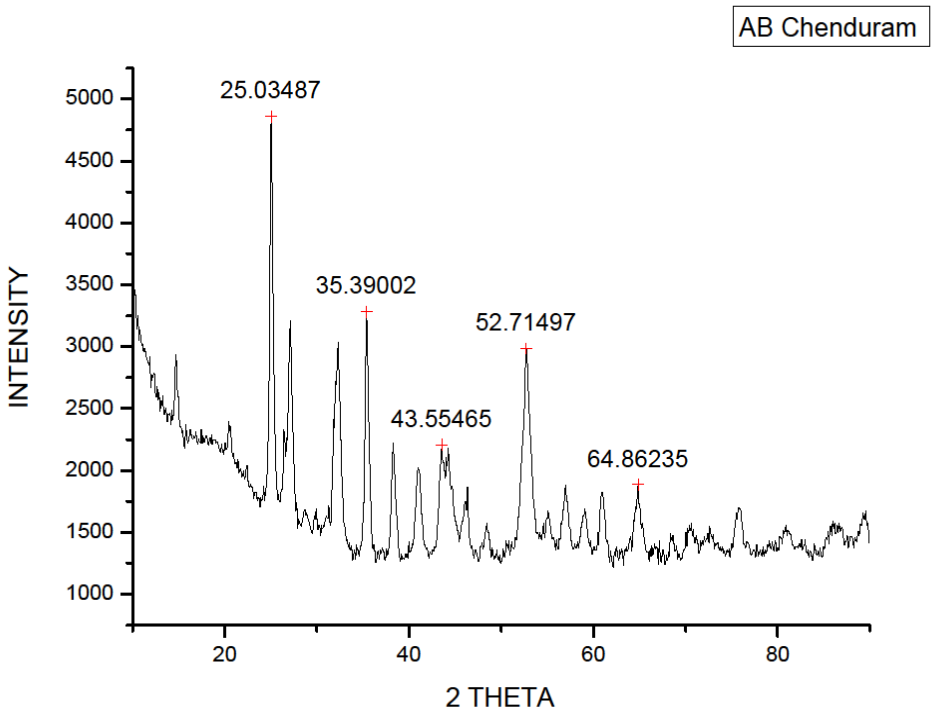


Figure: 13 Graph image of the drug by XRD analysis

DISSCUSSION:

XRD pattern of *AshtaBairavaChenduram* shows the good crystalinity after calcination process. The major diffraction peaks are identified after XRD analysis. *AshtaBairavaChenduram* concluded that HgS in nanocrystalline range (31–56 nm), in association with organic molecules probably plays an important role in making it biocompatible and nontoxic at therapeutic doses. Other elements present in *AshtaBairavaChenduram* act as additional supplement and possibly help in increasing the efficacy of the formulation. [108]

Toxicological study:

Acute oral toxicity study:

Table: 13.Dose finding experiment and its behavioral Signs of Toxicity for *ABC*

Group	Day
Body weight	**Normal**
Assessments of posture	**Normal**
Signs of convulsion limb paralysis	**Absence**
Body tone	**Normal**
Lacrimation	**Absence**
Salivation	**Absence**
Change in skin colour	**Normal**
Piloerection	**Normal**
Defecation	**Normal**
Sensitivity response	**Normal**
Locomotion	**Normal**
Muscle gripness	**Normal**
Rearing	**Normal**
Urination	**Normal**

Table: 14. Observation done:

Dose mg/ kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-

1.Alertness 2.Aggressiveness 3. Pile erection 4. Grooming 5.Gripping 6. Touch Response 7. Decreased Motor Activity 8.Tremors 9 Convulsions 10. Muscle Spasm 11. Catatonia 12.Musclerelaxant 13.Hypnosis 14.Analgesia15.Lacrimation 16. Exophthalmos 17. Diarrhoea 18. Writhing 19 Respiration 20. Mortality

Acute toxicity:

In the acute toxicity study, the rats were treated with different concentration of ***ASHTA BIRAVA CHENDHURAM*** from the range of 5mg/kg to 200mg/kg which did not produce signs of toxicity, behavioral changes, and mortality in the test groups as compared to the controls when observed during 14 days of the acute toxicity experimental period. These results showed that a single oral dose of the extract showed no mortality of these rats even under higher dosage levels indicating the high margin of safety of this extract. In acute toxicity test the ***ASHTA BIRAVA CHENDHURAM*** was found to be non- toxic at the dose level of 200mg/ kg body weight. [109]

Sub-Acute toxicity test

The dose selected for the sub-acute toxicity study was 20mg, 40mg/kg of ***ASHTA BIRAVA CHENDHURAM***. All the animals were free of intoxicating signs throughout the dosing period of 28 days. No physical changes were observed throughout the dosing period. No mortality was observed during the whole experiment. No abnormal deviations were observed. No significant changes were observed in the values of different parameters studied when compared with controls and values obtained were within normal biological and laboratory limits. The weights of organs recorded did not show any significant differences in the treatment and the

control group indicating that ***ASHTABIRAVA CHENDHURAM*** was not toxic to kidney, liver and spleen There was no significant changes were observed in hemoglobin (Hb), red blood cell (RBC), white blood cell (WBC), packed cell volume (PCV), Erythrocyte sedimentation rate (ESR) in all the treated groups as compared to respective control groups. Histopathology studies were carried out on liver, kidney and spleen and recorded. [110]

SUB ACUTE REPORTS :*ASHTA BIRAVA CHENDHURAM*

Sub-acute oral toxicity 28-days repeated dose study

Table.15.Body weight change of rat exposed to *ABC*

Dose (mg/kg/day)	**Days**				
	1	**7**	**14**	**21**	**28**
Control	**120.59±0.92**	**122.79±0.87**	**123.52±1.82**	**127.29±1.12**	**131.25±1.05**
20	**125.45±1.13**	**125.48±1.85**	**123.97±2.78**	**128.19±1.17**	**129.47±2.48**
40	**126.00±1.83**	**129.04±2.24**	**132.09±1.26**	**133.21±1.74**	**135.09±1.14**

Values are mean of 3 animals ± S.E.M

Table: 16 Effect of *ABC* on Organ weight in rats

ORGAN	**CONTROL**	**20 mg/kg**	**40 mg/kg**
Liver(g)	3.07±**0.20**	3.27±**0.11**	2.76±**0.08**
heart(g)	0.32±**0.04**	0.48±**0.01**	0.532±**0.52**
lung(g)	0.28±**0.05**	0.34±**0.04**	0.4±**0.06**
spleen(g)	0.25±**0.06**	0.31±**0.07**	0.35±**0.08**
Brain(g)	0.37±**0.05**	0.50±**0.05**	0.05±**0.03**
kidney(g)	0.76±**0.05**	0.93±**0.02**	0.73±**0.04**

*\Values are mean of 3 animals ± S.E.M. (Dunnett's test). *P<0.05; **P<0.01;***P<0.001 vs control N=3*

Table: 17. Effect of *ABC* on Haematological parameters in rats

Parameter	Control	15 mg/kg	30 mg/kg
RBC ($x 10^6/mm^3$)	8.29 ± 0.43	8.05±0.53	8.05±0.53
PCV (%)	49.66 ± 0.77	48.49±1.00	50.36±0.72
Hb (%)	15.13 ± 0.19	14.6±0.23	12.93±0.28
WBC ($x 10^3/mm^3$)	11.75 ± 0.85	12.06±1.04	14.27±0.34
Neutrophils (%)	23.29 ± 0.73	23.27±0.76	24.04±0.58
Lymphocytes (%)	85.5 ± 0.46	85.9±0.50	88.14±0.58
Eosinophils (%)	4.10 ± 0.23	4.1±0.32	3.26±0.32
Platelets ($x 10^3/mm^3$)	425.73± 1.35	463.8±16.9	513.9±13.9

*Values are mean of 3 animals ± S.E.M. (Dunnett's test). *P<0.05; **P<0.01;***P<0.001 N=3*

Table: 18.Effect of *ABC* on biochemical parameters in rats

Parameters	Control	15 mg/kg	30 mg/kg
Glucose (g/dl)	108.63 ± 0.81	111.08±0.9	108.3±0.47
Albumin (g/dl)	5.34 ± 0.40	5.65±0.71	6.98±0.53
BUN (mg/dl)	22.06± 1.55	22.72±1.25	24.2±1.11
Creatinine (mg/dl)	0.85 ± 0.07	0.85±0.04	0.96±0.02
Total Cholesterol (mg/dl)	93.21± 1.16	93.8±1.50	93.03±1.15
Triglycerides(mg/ dl)	52.58 ± 1.56	54.7±1.56	52.23±1.12
Total Protein (mg/dl)	8.58 ± 0.68	8.8±0.66	7.91±0.54

SGOT (U/L)	74.35 ± 1.23	74.6±1.70	70.13±0.57
SGPT(U/L)	27.07 ± 0.84	27.5±0.77	22.77±1.73
Alkaline phosphatase(U/L)	104.63 ± 1.14	105.2±1.19	99.9±0.65

*Values are mean of 3 animals ± S.E.M. (Dunnett's test). *P<0.05; **P<0.01;***P<0.001 N=3*

Table: 19.Effect of *ABC* on Urine parameters in rats

Parameters	Control	20 mg/kg	40 mg/kg
Colour	Yellow	Dark yellow	Brown
Transparency	Clear	Clear	Dark
Specific gravity	1.010	0.9	0.8
Ph	7.2	7.6	7.8
Protein	Nil	+	++
Glucose	Nil	-	-
Bilirubin	-ve	Trace	++
Ketones	-ve	-	-
Blood	Absent	Nil	Nil
RBCs	Nil	Nil	Nil
Epithelialcells	Nil	Nil	+
Casts	Nil	Occasional	+

Figure.15. Histopathology image of *ABC*

Results of In-Vitro Anti-Oral Cancer Activity

Table: 20.*Cytotoxicity Assay by MTT*

Sample Concentration (µg/ml)	Average OD at 540nm	Percentage Viability
Control	**0.4764**	
6.25	0.4018	84.3409
12.5	0.3747	78.6524
25	0.3379	70.9278
50	0.3012	63.2242
100	0.2971	62.3635

LD_{50} values –140.6ug/ml (ED50plus software V 1.0)

MTT colorimetric method, also known, is a method for detecting cell survival and growth methods. This assay is based on the metabolic reduction of 3- (4, 5-dimethylthiazol-2-yl) -2, 5-difeniltetrazol (MTT) by mitochondrial enzyme succinate dehydrogenase in a colored compound blue (formazan), allowing to determine the functionality of the mitochondrial treated cells. This method has been widely used to measure survival and cell proliferation. The amount of living cells is proportional to the amount of formazan produced. Cell lines derived from NCCS, Pune were free from any kind of bacterial and fungalcontamination.

AshtaBairavaChenduram at different doses (6.25-100 µg in100 µl of 5% MEM) was administeredfor 24 hrs. It was found that the number of cells decreases as the dose increases and at approximately 50 µg/mldose of extract, 50% of the cells (KB cells) were less as compared to normal control asshown in figure(15). The percentage of cells viability was determined by calculating the O.D of treated against thecontrol. Reading optical density (OD) is performed in a spectrophotometer at a wavelength of 540 nm. Comparison values are made on a basis of 50% inhibition of growth (IC_{50}) in treated cells with specific agents.

Results are tabulated in Table-20 and graphically represented in Chart-1

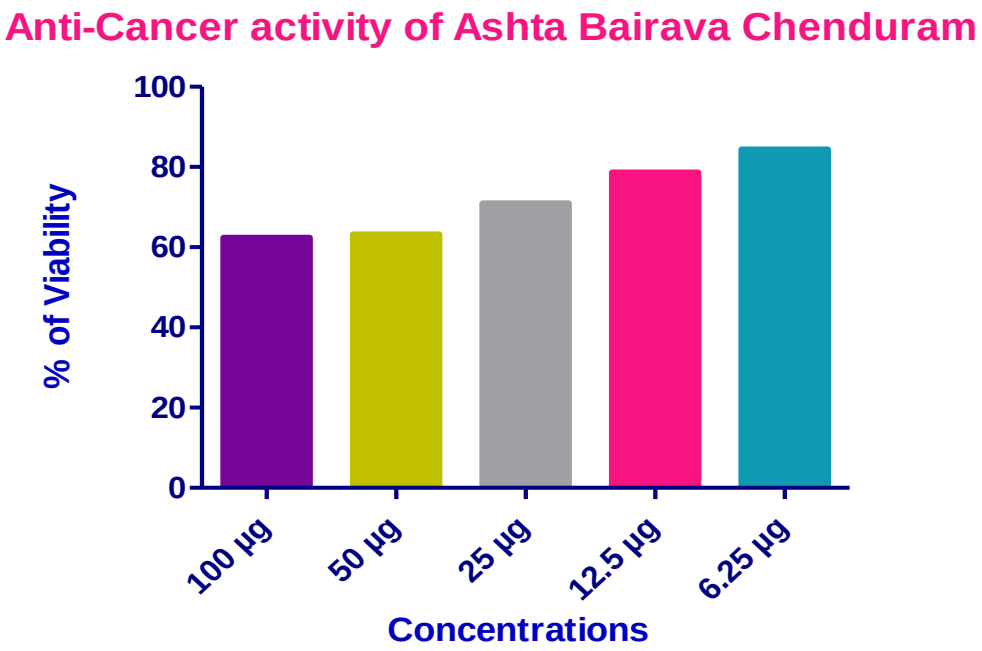


Chart: 1 Anti-Cancer activity of *ABC*

Chart -1shows the drug dose and % of Inhibition of KB cells after the *AshtaBairavaChenduram* extract treatment. It can be observed by the result of MTT assay that the IC dose of *AshtaBairavaChenduram* is 50µg/ml. As the dose increases the KB cell viability decreases. It was found that the % growth inhibition increasing with increasing concentration of *AshtaBairavaChenduram* steadily up to 6.25 µg / ml on *KB cell* line (Table-20 and Chart -1) and that IC value on *KB cell* line was 50 and R value was 0.9808.

Analysis of Membrane Morphological Characteristics by Haematoxylin /Eosin (H/E) Staining

Morphological changes such as changes to the cell membrane, loss of membrane asymmetry and cell shrinkage, are the early stage of apoptosis was analyzed by H/E staining. The IC dose (50µg/ml) treated cancer cells show features of apoptosis whereas treated with same amount of dose, to normal treated cells appeared without any significant changes.

Since the discovery of the cisplatin antitumor activity, great efforts have focused on the rational design of metal-based anticancer agents that can be potentially

used in cancer chemotherapy. Over the last four decades, a large number of metal complexes have been extensively investigated and evaluated *in vitro* and *in vivo.* [111]

The key focuses of these studies lie in finding novel metal complexes which could potentially overcome the hurdles of current clinical drugs including toxicity, resistance and other pharmacological deficiencies.

Metals and metal compounds have been used in medicine for several thousands of years. The medicinal uses and applications of metals and metal complexes are of increasing clinical and commercial importance. Monographs and major reviews, as well as dedicated volumes, testify to the growing importance of the discipline. [112]

The field of inorganic chemistry in medicine may usefully be divided into two main categories: firstly, ligands as drugs which target metal ions in some form, whether free or protein-bound; and secondly, metal-based drugs and imaging agents where the central metal ion is usually the key feature of the mechanism of action.

Arsenic trioxide, As_2O_3(Trisenox, Cell Therapeutics Inc, Seattle,USA) which was approved by the FDA in September 2000 for treatment of acute promomyelocyticleukemia (APL) in patients who have relapsed or are refractory to retinoid and anthracyclinechemotherapy. An estimated 1,500 new cases of APL are diagnosed yearly in the US, of which an estimated 400 patients will not respond to, or will relapse from, first-line therapy. [113]

The approval ofarsenic trioxide as a chemotherapeutic agent invokes the pioneering work of Ehrlich and thedevelopment of Salvarsan for use in syphilis—the foundation stone for the science of chemotherapy. [114]The use of chelating agents in medicine may even be traced to a collaboration between Werner (thefather of coordination chemistry) and Ehrlich (the father of chemotherapy) to find less toxic arseniccompounds for the treatment of syphilis. [115]

Arsenic has been used therapeutically for more than 2,000 years and was used in the 1930s for treatment of chronic myelogenousleukemia until supplanted by newer chemotherapies. [116][117]

The past, present, and future of medicinal arsenic has been described as a story of ‘‘use, dishonor, and redemption’’. Recent interest in arsenic trioxide initially arose through Chinese reports of its efficacy and use. Side effects are cardiotoxicity, skin rashes, and hyperglycemia. [118]

Arsenic trioxide apparently affects numerous intracellular signal transduction pathways and causes many alterations in cellular function. Thus, the mechanisms of cell death induced by arsenic trioxide are multiple; inductions of apoptosis, inhibition of proliferation, and even inhibition of angiogenesis have all been reported. [119][120]

In cellular studies, arsenic trioxide inhibits glutathione peroxidase, possibly through generation of arsenic–GSH conjugates, and increases cellular hydrogen peroxide content. [121]

Several pieces of evidence indicate that iron deprivation could be an excellent therapeutic approach: (i) dietary iron restriction markedly decreases tumour growth in rodents

and antibodies which block transferrin-binding to ellular receptors inhibit cancer cell growth *in vitro* and *invivo*.[122][123]

Sulphur is commonly used in Asia as an herbal medicine to treat inflammation and cancer. Organic sulphur has been studied on oral and other cancers and has been found to have remarkable benefit in anti-cancer therapy. [124]

Oncologists and scientists engaged in the research of cancer treatments should conduct a comprehensive study on the efficacy of mercury which is being used as an anti-cancer drug in the age old Siddha system. [125]

Three years of research has shown that metal (mercury, arsenic and copper) based Siddha drug is a safe alternative for cisplatin therapy or arsenic trioxide in selected cases of cancer treatments wherein the patients cannot bear the adverse effects. He found that mice treated with Siddha drugs showed better health than what did in cisplatin therapy in terms of appetite, haemoglobin, red blood cells and white blood cells. [126]

Studies have shown that phenols present in herbal plants such as *Acalyphaindica*, *Piper betel*, *Gossypiumherbaceum*, *Enicostemmaaxillare*, *Oimum sanctum* have cytotoxic effects on different tumors. Mechan+isms of these compounds are carried out through apoptosis. Thus from the above study, it is evident

that the cytotoxic property *of AshtaBairavaChenduram*may be due to the synergistic interactions between the metal complex and plant derivatives.

Figure: 15 -Morphological Effect Of *ABC* On Kb Cell Line

RESULT AND DISCUSSION

Anti- tumour activity

Cell apoptosis assay results

The dual parameter fluorescent dot plots (Fig -16) shows the viable cell population in quadrant 3 (negative annexin-FITC and negative PI), the cells at the early apoptosis are in quadrant 1 (positive annexin-FITC and negative PI) while the ones at the late apoptosis are in quadrant 2 (positive annexin-FITC and positive PI).As seen in fig :16 control untreated cells were mostly alive whereas when the *ABC* treatment was applied, the early apoptotic cells percentage increased in relation to its concentration. The box plot percentages of cell populations revealed that effect was dose and time dependent.

Figure: 16 Apoptotic effects of ABC on (a) OSCC and (b) HOS cell lines were determined by flow cytometry analysis using annexin V-FITC and propidium iodide. The experiments were performed after treatment with 2% and 5% ABC for 6, 12 and 24 hrs. Cell populations in bottom left, bottom right, top right and top left quadrants represented the proportion of viable cells, necrotic cells, late apoptotic cells and early apoptotic respectively.

DISCUSSION

In this study, we investigated the antiproliferative and apoptotic activities of ABC on human OSCC and HOS cell lines. The present study showed that ABC has potential time and dose dependent anti - proliferative effect on OSCC and HOS cancer cell lines. We found that ICfor ABC was 4% for OSCC and 3.5% for HOS cell lines. As shown by our flow cytometry results, when the concentration of ABC was increased, the percentage of early apoptotic cells also increased. For this reason, the mode of cell death appears to be due to early apoptosis cell death pathway.

Conclusion

In conclusion, the results of this study suggest that *ABC* has a promising anti- proliferative and apoptotic effect on OSCC and HOS cell lines. Early apoptosis could be attributed, in part, to its ability to inhibit proliferation. Further investigations are needed to determine the molecular mechanisms involved in apoptosis.

AntioxidantActivity

Table- 21.DPPHAssayof*AshtaBairavaChenduram*

Concentration (µg/µl)*	Absorbance		Percentage of inhibition	
AshtaBairavaChenduram	Drug	Standard	Drug	Standard
Control	0.9857	0.341	-	-
1.25	0.9693	0.299	20.78	40.89
2.50	0.9516	0.232	31.59	51.25
5.00	0.8839	0.114	43.45	74.07
10	0.7567	0.092	55.24	83.33
20	0.6392	0.054	62.12	89.62

µg/ml: microgram per millilitre. Drug: AshtaBairavaChenduram(1.25-20µg/µl). Standard: Ascorbic acid(10mg/mlDMSO)

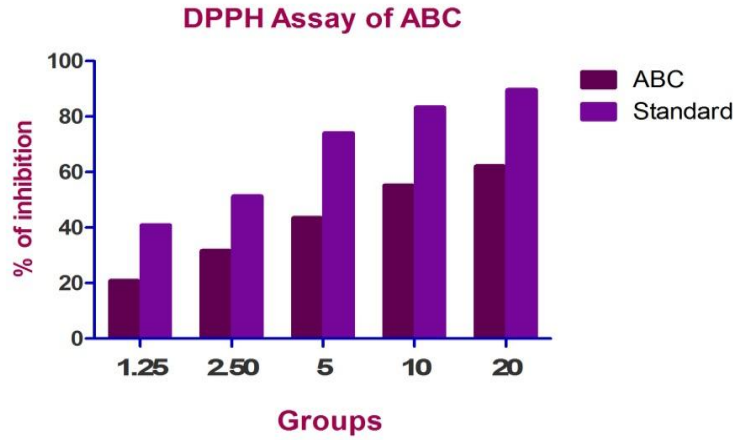


Chart: 2 DDPH Assay of *ABC*

DPPHstablefree radicalmethodis aneasy,rapidandsensitivewaytosurveytheantioxidant activity of*AshtaBairavaChenduram*extract. The antioxidant molecules can quench DPPH free radicals by providing hydrogen atomorbyelectron donation and a colorless stable molecule 1, 1 diphenyl-2-picrylhydrazilisformedandasaresultofwhichtheabsorbanceat517nmofthe solutionisdecreased.In thepresentstudy,the*AshtaBairavaChenduram*extractwasanalyzedwasableto decolorizeDPPH and thefreeradicalscavengingactivity wasexpressedasthepercentage decreaseinabsorbance.

The results show that the extracts of *AshtaBairavaChenduram* and the standard drug Ascorbic acid (10mg/mlDMSO) have a concentration-dependent anti-radical activity which was tabulated in Table No.21

A maximum of 62.12% and 89.62% anti-radical effects are exercised by *AshtaBairavaChenduram* and standard drug ascorbic acid at concentrations of 20 µg / ml respectively. Minimum percentageofinhibition20.78% and 40.89% % anti-radical effects are manifested by *AshtaBairavaChenduram*and standard drug ascorbic acid at concentrationsat 1.25µg/ml.Thisindicated that%ofinhibitionincreased withincreaseinconcentration ofboth thestandardand*AshtaBairavaChenduram*extract. But the*AshtaBairavaChenduram*extracthas lowerDPPHscavengingactivitythanthatof standard. From the present study, it was

concluded that *the AshtaBairavaChenduram*extracthasgood antioxidantactivityathigherconcentrations.

It is known that oxidative stress induced cell damage not only through damage to proteins, lipids and DNA. It may also alter signaling pathways redox sensitive to changes involved in the response of apoptosis. The antioxidants are currently the subject of many studies because, in addition to some interest in the preservation of comestibles, they could be useful in the prophylaxis and treatment of diseases in which oxidative stress is implicated. Many studies realized on natural products have proven that they are especially phenolic compounds who are responsible for their antioxidant activity.

Several studies have shown the link between the traditional drug formulations rich in antioxidants and the incidence certain diseases such as **cancer**, heart disease, diabetes and other diseases related to aging.Phenolic compounds could prevent cancer by the action antioxidant and / or the modulation of several functions of proteins. Phenolic compounds can prevent carcinogenesis by affecting the molecular events in the triggering, promotion and progression stages.

Some phenolic compounds (phenolic acids, flavonoids, quinones, coumarins) have proved an effective antioxidant activity and also had anticancer activities/ anticarcinogenic/ antimutagenic.

Here, the reactive oxygen species (ROS) may be the triggers apoptotic process. In recent years they have been described numerous properties of these compounds such as the ability to inhibit cell cycle, proliferation cellular and oxidative stress, and induce detoxification enzymes, apoptosis, and stimulate the immune system. It is therefore hypothesized that *AshtaBairavaChenduram* of its antioxidant power could “to repair" Cancer cellsti –oxidant activity

6. CONCLUSION

In the rat body weight examination it was found that as the days progressed the average weights of ascended up a little for controls and 15mg but weight descended gradually in mid and high dose group. However none of these were statistically significant, hence these variations can be ignored.

During organ weight examination there was a significant increase in kidney weight with the increase in dosage reaching high organ weight at 60mg/kg ($P<0.001$). Hence it has been advised to try this medicine with low and mid dosage.

In the examination of haematological and biochemical parameters there were notable changes in the experimental group when compared to control group. Though these variations are statistically significant all these changes are within normal limits.

Anti-cancer activity of the *ABC* is clearly visible from the MTT and DPPH assay. MTT assay result shows a high variation in cell viability of human oral cancer cell line. In addition it has been found cell viability variation increases with the increase in duration with the 15mg/ml dosage. This clearly shows the anticancer activity of the *ABC.*

In the DPPH assay the effectiveness in scavenging the free radical are measured. From the DPPH assay result we can conclude there is mean percentage reduction of free radicals during the dose level (15mg/ml) of *ABC.*

As a whole, haematological and biochemical variation are with in reference range. Body weight, organ weight and food consumption variations between control and experimental group were not significant. Anti-cancer activity of the *ABC* was well showed off from the MTT and DPPH assay.

From this we can conclude *ABC* is well tolerated by albino rats. Moreover considering all the above results *ABC* is not only found to be safer and without adverse effect it is also found to be more effective as an anti-cancer drug in minimal dose. This drug should be taken for further evaluation on a larger cohort. That will bring out an effective drug which would cast a bright light in the darkness of cancer therapy.

7.SUMMARY

The evaluation of therapeutic efficacy of the trial drug is "*AshtaBairavaChenduram*" from the Siddha literature "*Pranarakshamirthasinthu*" against Oral Carcinoma (*kannaputtru*) is dealt with in the entire study. Introduction of the study comprises about the prevalence of Carcinoma worldwide, which is found to be the major cause of the death in human. It is the right time to explore the therapeutic values of classic preparation against life threating malignancies to the society.

In the Drug Review,details about the therapeutic efficacy, active principles of Arsenic compounds and other ingredients and their role in the Siddha formulations indicated for Cancer are discussed.

SEM analysis revealed the size of the drug particle is in nanometer which implies that the drug could have potent drug delivery.

FTIR analysis found the presence of Phenolic groups which could act as an anti-oxidant and prevent classical risk factors free radicals of Cancer.

The acute and Sub-acute toxicity study exposed that the drug belonged to Class II and hence, we can use it for a long period.

So, based on all results it can be concluded that the anticancer activity of the drug in minimal dose in Oral Squamous Cell Carcinoma cell line proved it to be an effective anticancer drug and Lipid peroxidation - TBARS assay shown effective antioxidant activity.

All physicochemical analysis, elemental analysis and pharmacological evolution revealed that *AshtaBairavaChendooram* is an excellent traditional medicine in the management for Oral cancer.

The final discussion and conclusion parts provide a brief description of the entire analysis of study. The conclusion chapter also provides a discussion of the verification and validity of the research results carried out. The most vital part of some experience of the findings in the dissertation is also discussed and thereafter invites anyone who is ambitious to further studies and future research possibilities.

8.FUTURE SCOPE

The trial drug *Ashta Bairava Chenduram* is a potent herbo-mineral formulation with anti - cancerous properties which has been elaborated in the study. The above study performed in cell lines demonstrates the effectiveness of the drug *Ashta Bairavan chenduram* against oral carcinoma. Further evaluation of potency of the drug has to be explicitly done pre-clinically and clinically in future.

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